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volume II

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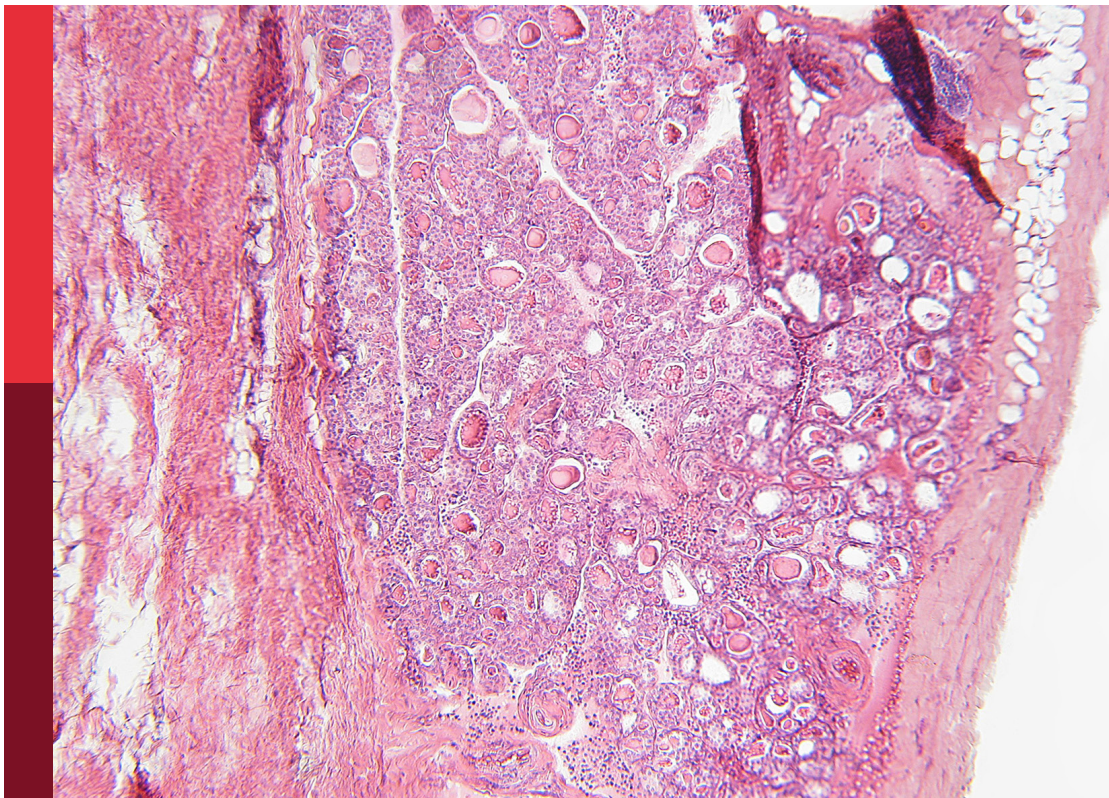

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What's new in endocrinology? volume II

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Insulin-like growth factors and aging: lessons from Laron syndrome

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The growth hormone (GH)-insulin-like growth factor-1 (IGF1) signaling pathway emerged in recent years as a key determinant of aging and longevity. Disruption of this network in different animal species, including flies, nematodes and mouse, was consistently associated with an extended lifespan. Epidemiological analyses have shown that patients with Laron syndrome (LS), the best-characterized disease under the umbrella of the congenital IGF1 deficiencies, seem to be protected from cancer. While aging and cancer, as a rule, are considered diametrically opposite processes, modern lines of evidence reinforce the notion that aging and cancer might, as a matter of fact, be regarded as divergent manifestations of identical biochemical and cellular underlying processes. While the effect of individual mutations on lifespan and health span is very difficult to assess, genome-wide screenings identified a number of differentially represented aging- and longevity-associated genes in patients with LS. The present review summarizes recent data that emerged from comprehensive analyses of LS patients and portrays a number of previously unrecognized targets for GH-IGF1 action. Our article sheds light on complex aging and longevity processes, with a particular emphasis on the role of the GH-IGF1 network in these mechanisms.

KEYWORDS

growth hormone (GH), insulin-like growth factor-1 (IGF1), IGF1 receptor, Laron syndrome, aging

Introduction to the GH-IGF1 system

Pituitary-derived growth hormone (GH) along with insulin-like growth factor-1 (IGF1) constitute an endocrine axis with critical roles in growth and development (1–3). The original hypothesis of Salmon and Daughaday, formulated in the late 1950s, claimed that the vast majority of the biological actions of GH are mediated by an hepatic peptide at first termed *somatomedin* and, subsequently, IGF1 (4). IGF1 is evolutionarily and structurally related to insulin. Prenatal IGF1 expression is GH-independent and becomes GH-dependent around the time of birth. After delivery, liver IGF1 production continues to be dependent on hypophysial GH secretion throughout all stages of life (5).

Aging is linked to various endocrine deficits. In the specific context of the somatotrophic axis, GH and IGF1 biosynthesis progressively decrease as we age due to reduced activity of the hypothalamic GH releasing hormone (GHRH)-GH neuroendocrine system (6). Thus, while maximal GH and IGF1 levels are reached at mid-puberty, concentrations around the eight decade of life become drastically reduced (7). Indeed, both the amplitude of the GH secretory pulses as well as the basal levels between pulses are largely decreased (8). Reduction of endocrine GH levels is closely followed by a parallel decline in circulating IGF1.

Evidence has accumulated in recent years demonstrating that disturbance of the GH-IGF1 network correlates with prolonged lifespan in a number of animal species, including flies (*D. melanogaster*), nematodes (*C. elegans*) and mouse (*M. musculus*) (9–11). Male mice harboring a disrupted GH receptor (*GHR*) gene ('*Laron*' mice) survive 55% longer than wild-type animals whereas female *Laron* mice have a 38% longer lifespan (12). The cellular and biochemical mechanisms that are responsible for the association between abrogation of the GH-IGF1 axis and prolonged lifespan are complex. Briefly, these mechanisms are functionally linked to the physiological role played by these hormones in nutrient sensing (13). Of relevance, whereas the effect of individual mutations on lifespan and health span in humans is usually difficult to assess, genomic analyses identified several differentially-represented aging-associated genes in Laron syndrome (LS) patients (14–16).

The present review article summarizes recent data concerning the linkage between the GH-IGF1 axis and aging. Our review highlights mechanistic aspects that emerge from genomic, bioinformatic and biochemical analyses of LS patients. These studies identified new, previously unrecognized targets for GH-IGF1 action and shed light on complex aging and longevity processes (17).

Laron syndrome

Growth retardation in children is linked to multiple factors and conditions. Cases in which no specific genetic, molecular or biochemical defect can be identified are regarded as *idiopathic* (5). *Congenital IGF1 deficiencies* are typically associated with low serum IGF1 but normal to high GH levels (18). IGF1 deficiencies may result from:

(1) GHRH-receptor (*GHRH-R*) defect;
(2) *GH* gene deletion (isolated GH deficiency, IGHD);
(3) GH receptor (*GHR*) gene deficiency (Laron syndrome, LS); and
(4) *IGF1* gene deletion.

Further conditions resulting in congenital IGF1 deficiency are deficiencies of post-GHR signaling (*e.g.*, *STAT5* defects), acid-labile subunit (*ALS*) mutations and pregnancy-associated plasma protein A2 (*PPA2*) mutations (19–24). Congenital IGF1 deficiencies provide an exceptional chance to address key physiological and pathological aspects of the GH-IGF1 axis. Even though these diseases are very rare, fundamental paradigms were derived from the analyses of these conditions, colloquially termed '*experiments of nature*' (3, 5, 25, 26).

Laron syndrome is the best described type of IGF1 deficiency under the spectrum of the GH-IGF1 pathologies (27). The main traits of LS children are short stature (-4 to -10 SDS below median), characteristic face, adiposity, elevated serum GH and low IGF1, insensitivity to GH administration (28–30). The identification of a mutated *GHR* gene as the etiological factor underlying LS was first reported in 1989 (31, 32). In subsequent studies, a series of *GHR* gene anomalies were identified (33). These defects included exon deletions and nonsense, frame shift and missense mutations. Regardless of the variations in the *GHR* defects detected, the outcomes in terms of phenotype were highly similar. Finally, broad analyses of the disease over more than fifty years have had a huge impact on our understanding of normal and pathological growth (16, 18, 34).

Laron syndrome and cancer protection

While a link between *high* IGF1 levels and enhanced cancer risk has been recognized more than twenty-five years ago, a potential protective role of *low* IGF1 dosages has been more difficult to demonstrate (35–38). This last concept has been supported by an epidemiological study conducted on a cohort of congenital IGF1 deficient patients, which revealed a marked reduction in cancer incidence in homozygous patients compared to their heterozygous relatives (39). The analysis included 230 LS patients, 116 patients with IGHD, 79 patients with *GHRH-R* defects, and 113 patients with congenital multiple pituitary hormone deficiency (cMPHD)]. In addition, the study included 752 of their first-degree family members. Among the 230 LS patients, not a single one developed cancer. Among the 116 IGHD patients, only one had a tumor. On the other hand, among first-degree family members (mostly heterozygotes) 30 instances of cancer were reported. Notwithstanding the fact that the total number of patients was modest, differences between patients and relatives were regarded as highly significant in statistical terms. Furthermore, while the total number of LS patients worldwide is unknown, it is estimated that the percentage of LS patients included in this epidemiological survey was 30-40% of the entire worldwide LS population (27, 30, 40).

The epidemiologic proof that patients with LS do not develop cancer is of foremost clinical relevance. This discovery is in agreement with the notion that the somatotrophic axis is of critical importance in the cell's '*decision*' whether to engage in proliferation or apoptosis (41, 42). Early studies have identified IGF1 as a progression factor that is required for cell cycle transition (43). Moreover, the bioactivities of IGF1 in the chain of events leading from a normal cell to a malignantly transformed one have been, to a large extent, dissected in biochemical terms. The neoplastic traits include: growth factor independence, chromosomal abnormalities, loss of cell-cell contact inhibition,

activation of oncogenes, accumulation of mutations, and others (44). The identification of pathways associated with IGF1 action ("*IGF1 signatures*") will have a great impact on the optimization of therapeutic tools directed against this growth factor system. Furthermore, these analyses will impinge on the ability to predict responsiveness to anti-IGF1R selective drugs (45–48).

Cancer protection and aging pathways exhibit a major overlap

As alluded to above, over the past decades the GH-IGF1 axis emerged as a critical determinant of aging and longevity. While cancer and aging are generally believed to constitute largely opposite processes, modern lines of evidence support the concept that cancer and aging might be regarded as different outcomes of the same fundamental processes. These processes include, among others, genomic instability, accumulation of cellular damage, *etc* (Table 1) (13).

While increased IGF1 levels as well as constitutive activation of the IGF1R are important risk factors in cancer, reduced activities of the GHR, IGF1R, insulin receptor and downstream mediators (*e.g.*, AKT, mTOR, FOXO) have been associated with a prolonged lifespan (49). Paradoxically, classical studies have shown an association between GH/IGF1 deficiency and a number of age-related features (50, 51). Some of these traits include thinning of the skin, excess adiposity, reduced muscle mass, reduced physical performance, *etc* (Table 2). The fact that features associated with GH deficiency, as detailed above, constitute manifestations of an aging archetype that is, intuitively, opposed to that classically correlated with enhanced longevity suggest the existence of complex underlying signaling networks. Divergent actions of GH and IGF1 might provide, at least in part, a biologically-plausible explanation to the diametrically opposite patterns of aging regulation depicted above. Furthermore, it is clear that it is not always feasible to infer from flies and nematode models into human biology (52). Hence, extreme care should be exerted when performing such extrapolations. The important role of the IGF1 axis in mitochondrial biology and oxidation processes and, particularly, the impact of these processes on senescence is described below.

TABLE 1 Common biochemical and cellular processes underlying cancer and aging.

Accumulation of cellular damage
Genomic instability
Epigenetic alterations
Deregulated nutrient sensing
Mitochondrial dysfunction
Stem cell exhaustion
Cellular senescence
Telomere attrition

TABLE 2 Resemblance between GH deficiency and aging.

Thinning of skin (wrinkling)
Excess of adipose tissue (obesity)
Decline in β-cell function
Enhanced insulin resistance (type 2 diabetes)
Reduced lean body mass (muscle reduction)
Reduced physical performance
Reduced mineral density (osteoporosis)
Elevated serum cholesterol

The GH-IGF1 axis and lifespan: studies in humans

The potential impact of the age-associated decrease in GH and IGF1 levels on lifespan and health span has been a matter of debate (53–55). Examination of prospective correlations of serum IGF1 with mortality, vascular disease, dementia, osteoporosis, diabetes and cancer, led to the identification of two general patterns (56). First, younger persons with high IGF1 are, for the most part, protected from disease. In contrast, older individuals with elevated IGF1 are at risk for occurrence of disease or death. Second, the correlation between IGF1 levels and disease risk is U-shaped. Hence, both high and low IGF1 concentrations might be harmful. Cancer, which is generally positively correlated with IGF1 levels, should be regarded as an exception to this U-shaped pattern. As a corollary, IGF1 signaling could be detrimental in older adults. Patients with LS who were not treated with IGF1 constitute a unique prototype for evaluating the impact of genetically low IGF1 on lifespan and health span (30). We can state with a high degree of confidence that lifelong IGF1 deficiency in untreated LS patients does not appear to noticeably prolong their lifespan. On the contrary, if their cardiovascular and metabolic problems are not treated in time, their lifespan might be shortened. In conclusion and despite the absence of definite epidemiological substantiation on longevity in congenital IGF1 deficiencies, the pivotal role of the GH-IGF1 network in the control of lifespan, as described above, has been extensively documented in various animal models.

Genomic analysis of LS patients identifies TXNIP as a novel IGF1 target gene linked to senescence regulation

Recently conducted genomic analyses of LS patients reported the identification of differentially expressed signaling pathways and genes in immortalized lymphoblastoid cells. Patients were compared to age-, gender- and ethnicity-matched controls (14, 15). Bioinformatics analyses allowed the clustering of differently expressed genes on the basis of their biological roles (Figure 1). Among other biological categories, fifteen percent of the identified genes participated in metabolism. Given the central regulatory role

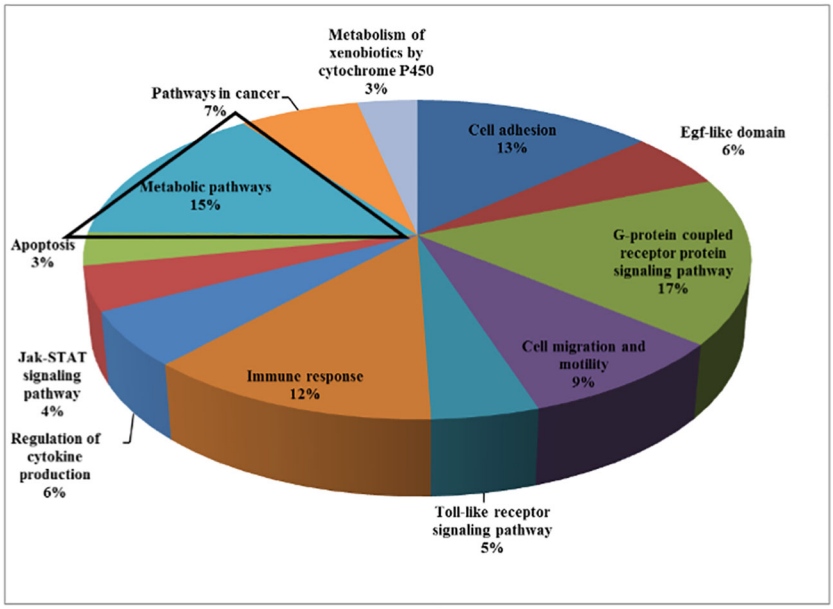

FIGURE 1
Genome-wide profiling of LS patients. Cluster analysis of differentially expressed genes in LS patients (n =4) compared to healthy controls (n = 3) of the same gender, age, and ethnic origin was conducted. Functional analyses were performed to find co-expressed genes sharing the same pathways. Analyses provide evidence for a number of shared pathways, including cell adhesion, G-protein signaling pathway, cell migration and motility, immune response, Jak-STAT signaling, apoptosis, etc. About 15% of the differentially expressed genes were involved in metabolic pathways. For the most part, genes involved in the control of cell cycle, motility, growth, and differentiation were downregulated in LS-derived lymphoblastoid cell lines compared with controls.

of IGF1 and insulin in the metabolism of cancerous cells, it is logic to assume that adjustments in the expression of metabolic genes may be mechanistically relevant towards the acquisition of a transformed phenotype (16).

The thioredoxin-interacting protein (TXNIP) was identified in genomic analyses as one of the top upregulated genes in LS. TXNIP (57) is an important player in several cellular processes, including metabolism and apoptosis (58–60). For example, TXNIP inhibits glucose uptake, with important consequences in terms of cell metabolism (61). TXNIP stabilizes p16 and p27, two Cdk inhibitors, with ensuing inhibition of cell division (62). Based on these activities, TXNIP is classified as a member of the cell cycle inhibitory enzymes. In agreement with this classification, downregulation of TXNIP is regarded as a prerequisite for cell division. Hence, TXNIP operates as a *bona fide* tumor suppressor (63–65).

Genomic analyses discovered a functional link between IGF1 and TXNIP (66, 67). Specifically, TXNIP was shown to be expressed at high levels in LS cells. Given that TXNIP has a key role in cellular redox regulation, and in view of the fact that IGF1 controls TXNIP levels under various stress situations (*e.g.*, high glucose, oxidative stress), we postulated that the IGF1-TXNIP loop has a crucial role in helping achieve an optimal balance in cellular homeostasis. Our data demonstrated that TXNIP is of vital importance for the cell fate choice, particularly when cells are confronted with different stress signals (Figure 2).

The cell state known as *cellular senescence* has been shown in recent years to be implicated in several physiological processes as well as in a number of age-related disorders (68–70). Senescence is usually tied to senescence-associated growth arrest, which is characterized by a senescence-associated secretory phenotype. Our studies have provided evidence that extended IGF1 treatment *in vitro* stimulates the acquisition of a premature senescence phenotype. This phenotype is typified by a unique senescence signature (67). Hence, IGF1 plays a dual role by stimulating mitosis and survival following short-term treatment while inducing premature senescence after long-term exposure (Figure 3).

Laron syndrome is associated with dysregulation of MIR132-3P: impact on aging genes

In addition to the transcriptional analyses depicted in the previous section, genome-wide surveys were conducted to identify microRNAs (miRs) that are differently expressed in LS. We hypothesized that differently represented miRs might account for, at least part of, the phenotypic traits of LS patients. MiRs are endogenous short non-coding RNAs that control the expression of complementary mRNAs (71–73). MiRs pair to specific protein-coding mRNAs, with ensuing post-transcriptional silencing of target genes. miRs are involved in multiple processes. These processes include cell death and proliferation, patterning of the nervous system and hematopoiesis. Finally, a number of miRs that are involved in the modulation of members of the IGF signaling pathway have been identified (74–76).

MiR-132-3p affects a number of biological functions (*e.g.*, inflammation, angiogenesis, neuronal differentiation, *etc*) and therefore is considered a key miR (77). Our analyses showed that

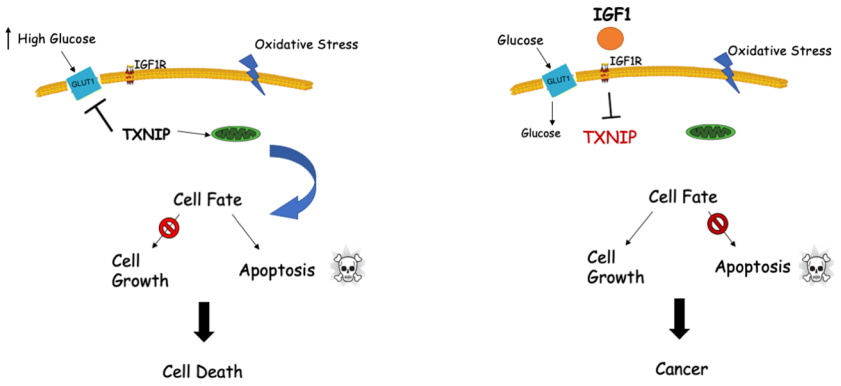

FIGURE 2
Interplay between IGF1 and TXNIP in regulation of cell survival and homeostasis. TXNIP was shown to be upregulated under normal physiological stress conditions like starvation, oxidative and glucose stresses. Upregulated TXNIP initiates apoptosis by interacting with thioredoxin and translocating to mitochondria (left panel). Cellular stress in the presence of IGF1 (right panel) might lead to marked downregulation of TXNIP levels with ensuing deregulated cell growth, including cancer.

miR-132-3p is highly expressed in LS. Given that LS is associated with low IGF1 levels, we postulated that miR-132-3p is negatively regulated by IGF1. Bioinformatics analyses helped identify a series of genes whose expression is modulated by miR-132-3p. Lastly, the mechanistic aspects of the IGF1-miR-132-3p regulatory loop are yet to be elucidated.

Using genome-wide analyses we identified SIRT1 as a target for inhibitory miR-132-3p control. These results are in accord with Hadar et al. (78), who reported a 4-fold lower expression of SIRT1 and a higher expression of miR-132 in Alzheimer's disease patients. SIRT1 is a member of the sirtuins family, a group of mammalian class III histone deacetylases. Sirtuins were mainly investigated in the context of health span and longevity. SIRT1 controls mitochondrial, endocrine and hypothalamic functions (79–82). In addition, SIRT1 is involved in memory formation in the brain by promoting axonal elongation and dendritic branching and by modulating synaptic plasticity (83). Of particular relevance, SIRT1 has been widely investigated in the context of longevity and neuroprotection. Taken together, the identification of SIRT1 as a downstream target for miR-132-3p provides the physical foundation for the link between disruption of the GH-IGF1 axis and prolonged lifespan (84).

Conclusions

The GH-IGF1 endocrine system has a critical role in determining lifespan, longevity and aging processes. We

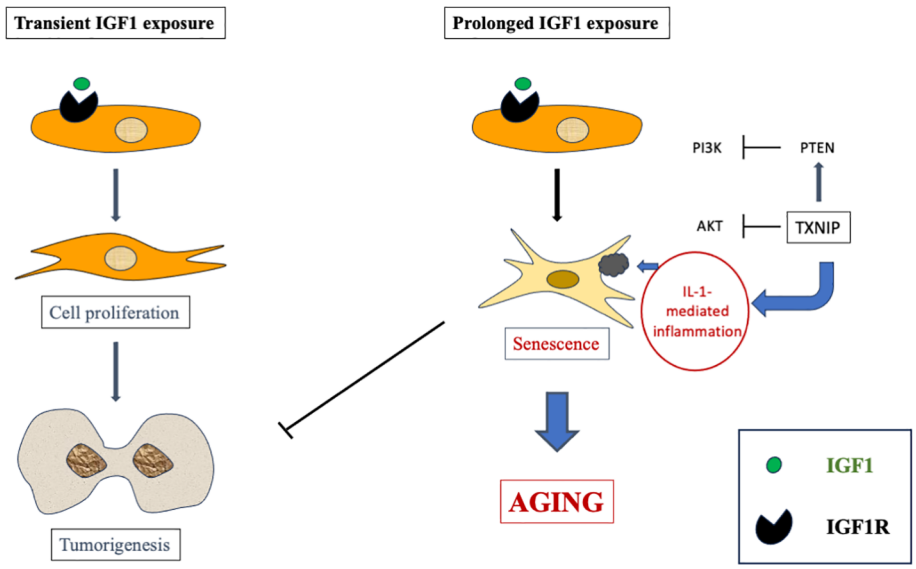

FIGURE 3
Schematic representation of short- versus long-term IGF1 treatment. Whereas short-term IGF1 stimulation is usually associated with cell proliferation and, potentially, tumorigenesis, prolonged IGF1 stimulation leads to cellular senescence via interaction with mitochondrial protein TXNIP.

postulated that life-long deficiency of IGF1 in LS might activate cancer-protecting pathways at the organismal level, including apoptotic and autophagic mechanisms. In parallel, diminished IGF1 signaling might have a significant impact on nutrient sensing and response to oxidative stress, leading to an extended lifespan (*at least* in animal models). Our comprehensive analyses have identified a number of new targets for IGF1 action whose over- or under-representation in LS might be linked to cancer evasion and, possibly, extended lifespan. The worldwide dispersion of the small number of patients with genetic IGF1 deficiency hinders to reach a definite conclusion.

The identification of miR-132-3p as a top upregulated miR in LS is of major interest. We may envision a scenario in which low IGF1 concentrations in patients lead to enhanced miR-132-3p levels. In turn, this specific miR is directly responsible for SIRT1 inhibition and, most probably, additional gene expression. The transcriptional and epigenetic mechanisms that control the concerted expression of the IGF1-miR-132-3p-SIRT1 axis are yet to be dissected.

Finally, by mining genomic and epigenomic data from LS patients we might be able to generate new clinical information. This information will eventually translate into new avenues of research in the areas of aging, metabolism and oncology. We believe that our results may shed light on genetic and epigenetic events associated with increased lifespan in models of IGF1 deficiency. These studies might have a major translational impact in medicine.

Author contributions

HW: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Project administration, Supervision, Writing – original draft, Writing – review and editing. ZL: Conceptualization, Investigation, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Yakar S, Werner H, Rosen CJ. Insulin-like growth factors: actions on the skeleton. *J Mol Endocrinol* (2018) 61:T115–T37. doi: 10.1530/JME-17-0298

2. LeRoith D, Yakar S. Mechanisms of disease: metabolic effects of growth hormone and insulin-like growth factor-1. *Nat Clin Pract Endocrinol Metab* (2007) 3:302–10. doi: 10.1038/ncpendmet0427

3. Rosenfeld RG. Insulin-like growth factors and the basis of growth. *New Engl J Med* (2003) 349:2184–6. doi: 10.1056/NEJMp038156

4. Salmon WD, Daughaday WH. A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in *vitro*. *J Lab Clin Med* (1957) 49:825–36.

5. Klammt J, Pfaffle R, Werner H, Kiess W. IGF signaling defects as causes of growth failure and IUGR. *Trends Endocrinol Metab* (2008) 19:197–205. doi: 10.1016/j.tem.2008.03.003

6. Yang J, Anzo M, Cohen P. Control of aging and longevity by IGF-I signaling. *Exp Gerontol* (2005) 40:867–72. doi: 10.1016/j.exger.2005.08.001

7. Cappola AR, Auchus RJ, El-Hajj Fuleihan G, Handelsman DJ, Kalyani RR, McClung M, et al. Hormones and aging: an endocrine society scientific statement. *J Clin Endocrinol Metab* (2023) 108:1835–74. doi: 10.1210/clinem/dgad225

8. Nass R, Farhy LS, Liu J, Pezzoli SS, Johnson ML, Gaylinn BD, et al. Age-dependent decline in acyl-ghrelin concentrations and reduced association of acyl-ghrelin and growth hormone in healthy older adults. *J Clin Endocrinol Metab* (2014) 99:602–8. doi: 10.1210/jc.2013-3158

9. Kimura KD, Tissenbaum HA, Liu Y, Ruvkun G. daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans. *Science* (1997) 277:942–6. doi: 10.1126/science.277.5328.942

10. Kenyon C. A conserved regulatory system for aging. *Cell* (2001) 105:165–8. doi: 10.1016/S0092-8674(01)00306-3

11. Clancy DJ, Gems D, Harshman LG, Oldham S, Stocker H, Hafen E, et al. Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein. *Science* (2001) 292:104–6. doi: 10.1126/science.1057991

12. Coschigano KT, Clemmons D, Bellush LL, Kopchick JJ. Assessment of growth parameters and life span of GHR/BP gene-disrupted mice. *Endocrinology* (2000) 141:2608–13. doi: 10.1210/endo.141.7.7586

13. Lopez-Otin C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. *Cell* (2013) 153:1194–217. doi: 10.1016/j.cell.2013.05.039

14. Lapkina-Gendler L, Rotem I, Pasmanik-Chor M, Gurwitz D, Sarfstein R, Laron Z, et al. Identification of signaling pathways associated with cancer protection in Laron syndrome. *Endocrine Related Cancer* (2016) 23:399–410. doi: 10.1530/ERC-16-0054

15. Werner H, Lapkina-Gendler L, Achlaug L, Nagaraj K, Somri L, Yaron-Saminsky D, et al. Genome-wide profiling of Laron Syndrome patients identifies novel cancer protection pathways. *Cells* (2019) 8:596. doi: 10.3390/cells8060596

16. Werner H, Sarfstein R, Nagaraj K, Laron Z. Laron syndrome research paves the way for new insights in oncological investigation. *Cells* (2020) 9:2446. doi: 10.3390/cells9112446

17. Deelen J, Uh HW, Monajemi R, van Heemst D, Thijssen PE, Böhringer S, et al. Gene set analysis of GWAS data for human longevity highlights the relevance of the insulin/IGF-1 signaling and telomere maintenance pathways. *Age (Dordr)* (2013) 35:235–49. doi: 10.1007/s11357-011-9340-3

18. Rosenfeld RG. The molecular basis of idiophatic short stature. *Growth Hormone IGF Res* (2005) 15 Suppl A:S3–5. doi: 10.1016/j.ghir.2005.06.014

19. Cohen P, Rogol AD, Deal CL, Saenger P, Reiter EO, Ross JL, et al. Consensus statement on the diagnosis and treatment of children with idiopathic short stature: a summary of the Growth Hormone Research Society, The Lawson Wilkins Pediatric Endocrine Society and the European Society for Pediatric Endocrinology Workshop. *J Clin Endocrinol Metab* (2008) 93:4210–7. doi: 10.1210/jc.2008-0509

20. Woods KA, Camacho-Hubner C, Savage MO, Clark AJL. Intrauterine growth retardation and postnatal growth failure associated with deletion of the insulin-like growth factor I gene. *New Engl J Med* (1996) 335:1363–7. doi: 10.1056/NEJM199610313351805

21. Kofoed EM, Hwa V, Little B, Woods KA, Buckway CK, Tsubaki J, et al. Growth hormone insensitivity associated with a STAT5b mutation. *New Engl J Med* (2003) 349:1139–47. doi: 10.1056/NEJMoa022926

22. Domene H, Bengolea SV, Martinez AS, Ropelato MG, Pennisi P, Scaglia P, et al. Deficiency of the circulating IGF system associated with inactivation of the acid-labile subunit gene. *New Engl J Med* (2004) 350:570–7. doi: 10.1056/NEJMoa013100

23. Dauber A, Muñoz-Calvo MT, Barrios V, Domené HM, Kloverpris S, Serra-Juhé C, et al. Mutations in pregnancy-associated plasma protein A2 cause short stature due to low IGF-I availability EMBO Mol. *Med* (2016) 8:363–74. doi: 10.15252/emmm.201506106

24. Giacomozzi C, Martin A, Fernández MC, Gutiérrez M, Iascone M, Domené HM, et al. Novel insulin-like growth factor 1 gene mutation: broadening of the phenotype and implications for insulin resistance. *J Clin Endocrinol Metab* (2023) 17:1355–69. doi: 10.1210/clinem/dgac738

25. Domené S, Domené HM. Genetic mutations in the GH/IGF axis. *Pediatr Endocrinol Rev* (2018) 16:39–62. doi: 10.17458/per.vol16.2018.dd.geneticmutationsghigf

26. Domené S, Scaglia PA, Gutiérrez ML, Domené HM. Applying bioinformatic platforms, in *vitro*, and in *vivo* functional assays in the characterization of genetic variants in the GH/IGF pathway affecting growth and development. *Cells* (2021) 10:2063. doi: 10.3390/cells10082063

27. Laron Z, Kopchik JJ. *Laron Syndrome – from Man to Mouse*. Heidelberg: Springer-Verlag (2011).

28. Laron Z. Extensive personal experience. Laron syndrome (primary growth hormone resistance or insensitivity): the personal experience 1958-2003. *J Clin Endocrinol Metab* (2004) 89:1031–44. doi: 10.1210/jc.2003-031033

29. Laron Z, Pertzelan A, Mannheimer S. Genetic pituitary dwarfism with high serum concentration of growth hormone-a new inborn error of metabolism? *Isr J Med Sci* (1966) 2:152–5.

30. Laron Z, Kauli R, Lapkina L, Werner H. IGF-I deficiency, longevity and cancer protection of patients with Laron syndrome. *Mutat Res Rev Mutat Res* (2017) 772:123–33. doi: 10.1016/j.mrrev.2016.08.002

31. Amselem S, Duquesnoy P, Attree O, Novelli G, Bousnina S, Postel-Vinay MC, et al. Laron dwarfism and mutations of the growth hormone-receptor gene. *New Engl J Med* (1989) 321:989–95. doi: 10.1056/NEJM198910123211501

32. Godowski PJ, Leung DW, Meacham LR, Galgani JP, Hellmiss R, Keret R, et al. Characterization of the human growth hormone receptor gene and demonstration of a partial gene deletion in two patients with Laron-type dwarfism. *Proc Natl Acad Sci USA* (1989) 86:8083–7. doi: 10.1073/pnas.86.20.8083

33. Shevah O, Laron Z. Genetic analysis of the pedigrees and molecular defects of the GH-receptor gene in the Israeli cohort of patients with Laron syndrome. *Pediatr Endocrinol Rev* (2006) Suppl. 3:489–97.

34. Laron Z. Lessons from 50 years of study of Laron syndrome. *Endocr Pract* (2015) 21:1395–402. doi: 10.4158/EP15939.RA

35. Hankinson SE, Willett WC, Colditz GA, Hunter DJ, Michaud DS, Deroo B, et al. Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. *Lancet* (1998) 351:1393–6. doi: 10.1016/S0140-6736(97)10384-1

36. Chan JM, Stampfer MJ, Giovannucci E, Gann PH, Ma J, Wilkinson P, et al. Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. *Science* (1998) 279:563–6. doi: 10.1126/science.279.5350.563

37. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. *Nat Rev Cancer* (2008) 8:915–28. doi: 10.1038/nrc2536

38. Renehan AG, Zwahlen M CM, O'Dwyer ST, Shalet SM, Egger M. Insulin-like growth factor-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis. *Lancet* (2004) 363:1346–53. doi: 10.1016/S0140-6736(04)16044-3

39. Steuerman R, Shevah O, Laron Z. Congenital IGF1 deficiency tends to confer protection against post-natal development of Malignancies. *Eur J Endocrinol* (2011) 164:485–9. doi: 10.1530/EJE-10-0859

40. Werner H, Lapkina-Gendler L, Laron Z. Fifty years on: New lessons from Laron syndrome. *Isr Med Assoc J* (2017) 19:6–7.

41. Baserga R. The decline and fall of the IGF-I receptor. *J Cell Physiol* (2013) 28:675–9. doi: 10.1002/jcp.24217

42. Werner H, Laron Z. Role of the GH-IGF1 system in progression of cancer. *Mol Cell Endocrinol* (2020) 518:111003. doi: 10.1016/j.mce.2020.111003

43. Dupont J, Pierre A, Froment P, Moreau C. The insulin-like growth factor axis in cell cycle pregression. *Horm Metab Res* (2003) 35:740–50. doi: 10.1055/s-2004-814162

44. Werner H, LeRoith D. Hallmarks of cancer: The insulin-like growth factors perspective. *Front Oncol* (2022) 12:1055589. doi: 10.3389/fonc.2022.1055589

45. Simpson A, Petnga W, Macaulay VM, Weyer-Czernilofsky U, Bogenrieder T. Insulin-like growth factor (IGF) pathway targeting in cancer: Role of the IGF axis and opportunities for future combination studies. *Target Oncol* (2017) 12:571–97. doi: 10.1007/s11523-017-0514-5

46. King H, Aleksic T, Haluska P, Macaulay VM. Can we unlock the potential of IGF-1R inhibition in cancer therapy? *Cancer Treat Rev* (2014) 40:1096–105. doi: 10.1016/j.ctrv.2014.07.004

47. Crudden C, Girnita A, Girnita L. Targeting the IGF-1R: the tale of the tortoise and the hare. *Front Endocrinol* (2015) 6:64. doi: 10.3389/fendo.2015.00064

48. Cox OT, O'Shea S, Tresse E, Bustamante-Garrido M, Kiran-Deevi R, O'Connor R. IGF-1 receptor and adhesion signaling: an important axis in determining cancer cell phenotype and therapy resistance. *Front Endocrinol* (2015) 6:106. doi: 10.3389/fendo.2015.00106

49. Barzilai N, Huffman DM, Muzumdar RH, Bartke A. The critical role of metabolic pathways in aging. *Diabetes* (2012) 61:1315–22. doi: 10.2337/db11-1300

50. Laron Z. Effects of growth hormone and insulin-like growth factor-1 deficiency on ageing and longevity. In: *Endocrine Facets of Ageing*. Chichester: John Wiley & Sons Ltd (2002). p. 125–42.

51. Toogood AA, Shalet SM. Ageing and growth hormone status. *Bailliere's Clin Endocrinol Metab* (1998) 12:281–96. doi: 10.1016/S0950-351X(98)80023-2

52. Anversa P. Aginh and longevity: The IGF-1 enigma. *Circ Res* (2005) 97:411–4. doi: 10.1161/01.RES.0000182212.09147.56

53. Burgers AMG, Biermasz NR, Schoones JW, Pereira AM, Renehan AG, Zwahlen M, et al. Meta-analysis and dose-response metaregression: circulating insulin-like growth factor I (IGF-I) and mortality. *J Clin Endocrinol Metab* (2011) 96:2912–20. doi: 10.1210/jc.2011-1377

54. Friedrich N, Haring R, Nauck M, Lüdemann J, Rosskopf D, Spilcke-Liss E, et al. Mortality and serum insulin-like growth factor (IGF)-I and IGF binding protein 3 concentrations. *J Clin Endocrinol Metab* (2009) 94:1732–9. doi: 10.1210/jc.2008-2138

55. Milman S, Huffman DM, Barzilai N. The somatotropic axis in human aging: framework for the current state of knowledge and future research. *Cell Metab* (2016) 23:980–9. doi: 10.1016/j.cmet.2016.05.014

56. Zhang WB, Ye K, Barzilai N, Milman S. The antagonistic pleiotropy of insulin-like growth factor 1. *Aging Cell* (2021) 313443 20(9):e13443. doi: 10.1111/acel.13443

57. Zhou J, Yu Q, Chng WJ. TXNIP (VDUP-1, TBP-2): a major redox regulator commonly suppressed in cancer by epigenetic mechanisms. *Int J Biochem Cell Biol* (2011) 43:1668–73. doi: 10.1016/j.biocel.2011.09.005

58. Patwari P, Higgins LJ, Chutkow WA, Yoshioka J, Lee RT. The interaction of thioredoxin with Txnip: Evidence for formation of a mixed disulfide by disulfide exchange. *J Biol Chem* (2006) 281:21884–91. doi: 10.1074/jbc.M600427200

59. Aitken CJ, Hodge JM, Nishinaka Y, Vaughan T, Yodoi J, Day CJ, et al. Regulation of human osteoclast differentiation by thioredoxin binding protein-2 and redox-sensitive signaling. *J Bone Mineral Res* (2004) 19:2057–64. doi: 10.1359/jbmr.040913

60. Goldberg SF, Miele ME, Hatta N, Takata M, Paquette-Straub C, Freedman LP, et al. Melanoma metastasis suppression by chromosome 6: evidence for a pathway regulated by CRSP3 and TXNIP. *Cancer Res* (2003) 63:432–40.

61. Chutkow WA, Patwari P, Yoshioka J, Lee RT. Thioredoxin-interacting protein (Txnip) is a critical regulator of hepatic glucose production. *J Biol Chem* (2008) 283:2397–406. doi: 10.1074/jbc.M708169200

62. Jeon JH, Lee KN, Hwang CY, Kwon KS, You KH, Choi I. Tumor suppressor VDUP1 increases p27(kip1) stability by inhibiting JAB1. *Cancer Res* (2005) 65:4485–9. doi: 10.1158/0008-5472.CAN-04-2271

63. Elgort MG, O'Shea JM, Jiang Y, Ayer DE. Transcriptional and translational downregulation of thioredoxin interacting protein is required for metabolic reprogramming during G(1). *Genes Cancer* (2010) 1:893–907. doi: 10.1177/1947601910389604

64. Chutkow WA, Lee RT. Thioredoxin regulates adipogenesis through thioredoxin-interacting protein (Txnip) protein stability. *J Biol Chem* (2011) 286:29139–45. doi: 10.1074/jbc.M111.267666

65. Park JW, Lee SH, Woo GH, Kwon HJ, Kim DY. Downregulation of TXNIP leads to high proliferative activity and estrogen-dependent cell growth in breast cancer. *Biochem Biophys Res Commun* (2018) 498:566–72. doi: 10.1016/j.bbrc.2018.03.020

66. Nagaraj K, Lapkina-Gendler L, Sarfstein R, Gurwitz D, Pasmanik-Chor M, Laron Z, et al. Identification of thioredoxin-interacting protein (TXNIP) as a downstream target for IGF1 action. *Proc Natl Acad Sci USA* (2018) 115:1045–50. doi: 10.1073/pnas.1715930115

67. Nagaraj K, Sarfstein R, Laron Z, Werner H. Long-term IGF1 stimulation leads to cellular senescence *via* functional interaction with the thioredoxin-interacting protein, TXNIP. *Cells* (2022) 11:3260. doi: 10.3390/cells11203260

68. Paez-Ribes M, González-Gualda E, Doherty GJ, Muñoz-Espín D. Targeting senescent cells in translational medicine. *EMBO Mol Med* (2019) 11:e10234. doi: 10.15252/emmm.201810234

69. Kirkland JL, Tchkonia T, Zhu Y, Niedernhofer LJ, Robbins PD. The clinical potential of senolytic drugs. *J Am Geriatr Soc* (2017) 65:2297–301. doi: 10.1111/jgs.14969

70. Karin O, Agrawal A, Porat Z, Krizhanovsky V, Alon U. Senescent cell turnover slows with age providing an explanation for the Gompertz law. *Nat Commun* (2019) 10:5495. doi: 10.1038/s41467-019-13192-4

71. Guarnieri DJ, DiLeone RJ. MicroRNAs: a new class of gene regulators. *Ann Med* (2008) 40:197–208. doi: 10.1080/07853890701771823

72. Zalts H, Shomron N. The impact of microRNAs on endocrinology. *Ped Endocrinol Rev* (2011) 8:354–62.

73. Hobert O. Gene regulation by transcription factors and microRNAs. *Science* (2008) 319:1785–6. doi: 10.1126/science.1151651

74. Jiang L, Liu X, Chen Z, Jin Y, Heidbreder CE, Kolokythas A, et al. MicroRNA-7 targets IGF1R (insulin-like growth factor 1 receptor) in tongue squamous cell carcinoma cells. *Biochem J* (2010) 432:199–205. doi: 10.1042/BJ20100859

75. McKinsey EL, Parrish JK, Irwin AE, Niemeyer BF, Kern HB, Birks DK, et al. A novel oncogenic mechanism in Ewing sarcoma involving IGF pathway targeting by EWS/Fli1-regulated microRNAs. *Oncogene* (2011) 30:4910–20. doi: 10.1038/onc.2011.197

76. Dobre M, Herlea V, Vladut C, Ciocirlan M, Balaban VD, Constantinescu G, et al. Dysregulation of miRNAs targeting the IGF-1R pathway in pancreatic ductal adenocarcinoma. *Cells* (2021) 10:1856. doi: 10.3390/cells10081856

77. Li D, Wang A, Liu X, Meisgen F, Grünler J, Botusan IR, et al. MicroRNA-132 enhances transition from inflammation to proliferation during wound healing. *J Clin Invest* (2015) 125:3008–26. doi: 10.1172/JCI79052

78. Hadar A, Milanesi E, Walczak M, Puzianowska-Kuźnicka M, Kuźnicki J, Squassina A, et al. SIRT1, miR-132 and miR-212 link human longevity to Alzheimer's Disease. *Sci Rep* (2018) 8:8465. doi: 10.1038/s41598-018-26547-6

79. Tang BL. Sirt1 and the mitochondria. *Mol Cells* (2016) 39:87–95. doi: 10.14348/molcells.2016.2318

80. Leite JA, Ghirotto B, Targhetta VP, de Lima J, Câmara NOS. Sirtuins as pharmacological targets in neurodegenerative and neuropsychiatric disorders. *Br J Pharmacol* (2022) 179:1496–511. doi: 10.1111/bph.15570

81. Strum JC, Johnson JH, Ward J, Xie H, Feild J, Hester A, et al. MicroRNA 132 regulates nutritional stress-induced chemokine production through repression of SirT1. *Mol Endocrinol* (2009) 23:1876–84. doi: 10.1210/me.2009-0117

82. Ye F, Wu A. The protective mechanism of SIRT1 in the regulation of mitochondrial biogenesis and mitochondrial autophagy in Alzheimer's Disease. *J Alzheimers Dis* (2021) 82:149–57. doi: 10.3233/JAD-210132

83. Mishra P, Mittal AK, Kalonia H, Madan S, Ghosh S, Sinha JK, et al. SIRT1 promotes neuronal fortification in neurodegenerative diseases through attenuation of pathological hallmarks and enhancement of cellular lifespan. *Curr Neuropharmacol* (2021) 19:1019–37. doi: 10.2174/1570159X18666200729111744

84. Tran D, Bergholz J, Zhang H, He H, Wang Y, Zhang Y, et al. Insulin-like growth factor-1 regulates the SIRT1-p53 pathway in cellular senescence. *Aging Cell* (2014) 13:669–78. doi: 10.1111/acel.12219

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Multi-omics subgroups associated with glycaemic deterioration in type 2 diabetes: an IMI-RHAPSODY Study

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Introduction: Type 2 diabetes (T2D) onset, progression and outcomes differ substantially between individuals. Multi-omics analyses may allow a deeper understanding of these differences and ultimately facilitate personalised treatments. Here, in an unsupervised "bottom-up" approach, we attempt to group T2D patients based solely on -omics data generated from plasma.

Methods: Circulating plasma lipidomic and proteomic data from two independent clinical cohorts, Hoorn Diabetes Care System (DCS) and Genetics of Diabetes Audit and Research in Tayside Scotland (GoDARTS), were analysed using Similarity Network Fusion. The resulting patient network was analysed with Logistic and Cox regression modelling to explore relationships between plasma -omic profiles and clinical characteristics.

Results: From a total of 1,134 subjects in the two cohorts, levels of 180 circulating plasma lipids and 1195 proteins were used to separate patients into two subgroups. These differed in terms of glycaemic deterioration (Hazard Ratio=0.56;0.73), insulin sensitivity and secretion (C-peptide, p=3.7e-11;2.5e-06, DCS and GoDARTS, respectively; Homeostatic model assessment 2 (HOMA2)-B; -IR; -S, p=0.0008;4.2e-11;1.1e-09, only in DCS). The main molecular signatures separating the two groups included triacylglycerols, sphingomyelin, testican-1 and interleukin 18 receptor.

Conclusions: Using an unsupervised network-based fusion method on plasma lipidomics and proteomics data from two independent cohorts, we were able to identify two subgroups of T2D patients differing in terms of disease severity. The molecular signatures identified within these subgroups provide insights into disease mechanisms and possibly new prognostic markers for T2D.

KEYWORDS

multi-omics, type 2 diabetes, glycaemic deterioration, metabolic syndrome, lipidomics, proteomics

1 Introduction

It is increasingly recognised that type 2 diabetes (T2D) presentation differs markedly between patients (1). Nevertheless, approaches using current clinical measurements and classical laboratory markers are likely to be underpowered to capture the underlying heterogeneity within the disease (2). Better characterization of T2D at the molecular level is likely to be essential to move from a "one size fits all" approach to more precise health management of this disease (3).

Compared to traditional approaches, unbiased "multi-omics" analysis could potentially identify reliable patient-specific biomarkers for disease progression. Previously, using three T2D cohorts, the Hoorn Diabetes Care System (DCS), Genetics of Diabetes Audit and Research in Tayside Scotland (GoDARTS) and All New Diabetics in Scania (ANDiS), encompassing 2,973 individuals across three molecular classes, metabolites, lipids, and proteins, we were able to identify several novel biomarkers for T2D progression and prevalence (4). Based on both Cox and Logistic regression models, this study scanned each biomarker's potential association with T2D glycaemic deterioration then established causal relationships for some of these identified proteins, such as the Reticulon-4 receptor (NogoR/RTN4R) and the Interleukin-18 receptor 1 (IL18R1).

A limitation of this early study was that no account was taken for potential heterogeneity in disease status (5) which may confound the identification of biomarkers (whose association with disease may vary between sub-clusters). On the other hand, sub-clusters based on a limited number of clinical variables alone may be somewhat "artificial" or subjective in terms of molecular aetiology. Indeed, others (6, 7) have proposed that positioning individuals within a multi-dimensional continuum of biomarkers that globally reflect underlying disease progression or pathology may provide a useful prognostic strategy and better health management.

In the present study, we address these limitations by deploying an unsupervised, bottom-up approach for T2D individual allocation without any pre-assumptions based on clinical characteristics. Thus, we assess the relationships with disease progression and the circulating levels of a large number of molecules belonging to two classes, lipids and proteins.

We have interrogated data from two independent European T2D cohorts, DCS and GoDARTS, within the RHAPSODY consortium (8). T2D Patients with complete lipidomics (180 circulating lipids) and proteomics (1195 circulating proteins) data were queried and analysed through a non-disclosive federated infrastructure (9) using Similarity Network Fusion (SNF) (10). SNF combines different data modalities from the same patients into a similarity network, enabling patient clustering and subsequent feature extraction. This approach reveals the existence of two sub-clusters of individuals in each cohort, with distinct biomarker profiles and clinical characteristics.

2 Methods

2.1 Study populations

We used data from two type 2 diabetes cohorts: Hoorn Diabetes Care System (DCS) and Genetics of Diabetes Audit and Research in Tayside Scotland (GoDARTS) within the RHAPSODY consortium. RHAPSODY (Risk Assessment and Progression of Diabetes, https://imi-rhapsody.eu) is an Innovative Medicine Initiative project with the goal of enhancing the categorization of individuals with type 2 diabetes and aiding the adoption of innovative approaches for diabetes prevention and treatment.

The DCS cohort recruits almost all T2D patients from 103 GPs in the West-Friesland region of the Netherlands. This prospective, regional cohort study started in 1998 and by 2017, held 12,673 T2D patients with a median of 0.7 years (IQR 0.2-3.7) after diagnosis (11). The study was approved by the Ethical Review Committee of the Vrije Universiteit University Medical Center, Amsterdam. Measurements were labelled anonymously. All laboratory measurements were done on samples taken in a fasted state.

Between 1996 and 2015, GoDARTS recruited 10149 type 2 diabetes patients from the Tayside region of Scotland. Patients in the GoDARTS cohort were not necessarily recruited at the time of diagnosis (12). The GoDARTS study was approved by the Tayside Medical Ethics Committee. All laboratory measurements were measured in a non-fasted state.

Lipidomics and peptidomics are available for a subset of T2D patients in both DCS and GoDARTS cohorts. These were selected with a blood sample close to diagnosis and median diabetes duration of 2.6 and 1.4 years respectively. These data were collected at baseline and generated as part of the RHAPSODY project (4). Of note, individuals were selected without taking into consideration pre-cluster assignments.

Further information on the cohort characteristics can be found in Slieker et al., 2021 (13).

2.2 Measurements

Informed consent was obtained from all participants. In DCS, Haemoglobin A1c was measured based on the turbidimetric inhibition immunoassay for haemolysed whole EDTA blood (Cobas c501, Roche Diagnostics, Mannheim, Germany). The levels of triglycerides, total cholesterol and HDL cholesterol were measured enzymatically (Cobas c501, Roche Diagnostics) (11, 12). In DCS and GoDARTS, C-peptide was measured on a DiaSorin Liaison (DiaSorin, Saluggia, Italy). Plasma lipids were determined using a QExactive mass spectrometer (Thermo Scientific), without assessment of the liproprotein particles from which lipids were derived. Plasma protein levels were measured on the SomaLogic SOMAscan platform (Boulder, Colorado, USA). More details on plasma lipidomics and proteomics measurements can be found in Slieker et al., 2023 (4).

2.3 Network-based clustering of -omics data

A federated database of T2D cohorts including DCS and GoDARTS has previously been set up as part of the RHAPSODY project. This system enables statistical and machine learning analysis to be performed on cohort data remotely without any disclosure of sensitive data (14–17). The federated database system was interrogated using the R statistical programming language (version 4.0.4). In both DCS and GoDARTS cohorts, patients with complete lipidomics and peptidomics were used for clustering and subsequent statistical analysis. Lipidomics and peptidomics values were centred to a mean value of 0 and a standard deviation of 1 in each cohort using the *dssScale* function in R (*dsSwissKnifeClient* package) (18). Euclidean distances between each pair of patients were then calculated based on the normalized lipidomics or peptidomics data by using *dist2* function from *dssSNF* (*dsSwissKnifeClient* package) (18). *dssSNF* is a wrapper function for *SNFtool* enabling the analysis to be performed on a remote server without sensitive data disclosure. Patient similarity matrices were generated from the Euclidean distance matrices using the *affinityMatrix* function with parameters of K (the number of nearest neighbours) equal to 20 and hyperparameter alpha equal to 5 using *dssSNF*. The patient similarity matrices were then fused using the *SNF* function of the *SNFtool* package (10) with T (number of iterations) equal to 20 using *dssSNF* and clustering was performed using the *spectralclustering* function of the *SNFtool* of *dsSwissKnifeClient* packages (10, 18) Silhouette widths of fused cluster patients were calculated from the similarity matrices using the *Silhouette_SimilarityMatrix* function (*CancerSubtypes* package) (19) to determine the optimal cluster number.

SNF models were validated by bootstrapping tests (n=1000 iterations) that compared to models using randomized Euclidean distance matrices by *randomizeMatrix* (*picante* package) (20). For each simulated model, the same parameters were used, and the number of clusters is set to correspond to the number of clusters in the study model. The significance of each cluster (P-value <=0.05) was calculated by assessing the frequency of achieving a model with an equal or greater mean local cluster coefficient for randomized data. Local cluster coefficient calculation was performed with unweighted, undirected adjacency matrices using the *transitivity* function in the *igraph* package (21). The adjacency matrices were generated from the similarity matrices with the top 2.5% similarity values set as 1 and the rest as 0.

Multi-block Common Dimensions analysis (ComDim) was performed using *federateComDim* from the dsMO package in R (v0.1.12; https://github.com/vanduttran/dsMO) using default parameters. ComDim reduces the lipidomics and proteomics data into a smaller number of dimensions with global and block components for each patient.

2.4 Statistical analysis

Logistic regression model was performed using the *ds.glm* function of the *dsBaseClient* (16) package to test differences between clusters for each molecule and clinical measurements. The cluster classification was treated as a dependent categorical variable with age, sex and BMI acting as covariates. Subsequently, for lipids, peptides and clinical measurements, each cluster was subset for the strongly associated (P-value <=0.05) features and the mean values of each group of features in each cluster were calculated. Mean values were used since, to protect the patient's identity, individual-level data cannot be downloaded from the federated database system. Several mean values for each feature from 5 or more patients were calculated based on the default patients' order in the remote server for each cluster. The feature values were normalized. Hierarchical clustering was then performed, and the results were visualized as heatmaps using the R package *gplots* (22).

Cox regression was performed using the *dssCoxph* function in the *dssSwissKnifeClient* package. Time to insulin requirement was defined as the length of time from diagnosis until an individual started insulin treatment for a period of more than six months, or alternatively as more than two independent HbA1c measurements greater than 69 mmol/mol (8.5%) at least three months apart and when ≥2 non-insulin glucose-lowering drugs were taken. From the diagnosis until the primary endpoint, no known glucose lowering agents were taken during the monitoring period. Hazard ratios for time to insulin treatment requirement within the clusters were calculated using Cox regression with age, sex and BMI as covariates.

Analyses were performed using the R statistical programming language (version 4.0.4) and Python (Pycharm 2022.2.1). The

Benjamini-Hochberg procedure was used to determine the false discovery rate correcting for multiple tests. Figures were produced using *gplots* (version 3.1.1), *ggplot2* (version 3.3.4), *igraph* (version 1.2.6), *circlize* (version 0.4.15), *seaborn* (version 0.12.1), and *matplotlib* (version 3.6.0). The graphical abstract was generated using Biorender.

2.5 Data availability statement

Started in 2016, within the RHAPSODY project, we federated 12 patient cohorts totalling over 68000 diabetes patients. We have collected and generated "multi-omics" and genetic data alongside clinical data. All the data have been harmonized and stored in a federated infrastructure to enable remote query and analysis. Data analysis through the federated database has been made possible through the R programming environment with federated analysis packages. Information about accessing the Rhapsody federated database can be found at: https://imi-rhapsody.eu/.

3 Results

3.1 Multi-omics analysis separates T2D patients into two subgroups

The initial question we wanted to ask was whether T2D patients could be robustly separated based on their combined plasma lipidomics/proteomics signatures and whether molecular differences could highlight novel circulating biomarkers associated with disease severity or progression. To do this, we used a data-driven network-based clustering strategy (SNF) on plasma lipidomics and peptidomics data from a total of 1,134 patients with T2D in two independent cohorts, for which baseline characteristics are given in Table 1. A total of 589 (DCS) and 545 (GoDARTS) patients with T2D, for whom complete plasma lipidomics and peptidomics datasets were available, were used for the analysis. The characteristics of the two cohorts were comparable in terms of average age and BMI, with a majority of males present in each case (13). Individual assignments were performed based on SNF for subgrouping similar multi-omics T2D patients.

TABLE 1 Characteristics of the individuals included from the two cohorts.

	DCS	GoDARTS
n	589	545
Male,%	56.7	59.3
Age, years	62 (55.3-69)	61 (53-69)
BMI, kg/m2	29.7 (26.9-33.1)	30.5 (27.2-35.0)
HbA1c, mmol/mol	47.5 (43.5-52.0)	53.0 (46.0-61.0)
C-peptide, nmol/l	1.1 (0.8-1.4)	2.0 (1.4-2.7)
HDL-cholesterol, mmol/l	1.2 (1.0-1.4)	1.3 (1.1-1.5)
LDL-cholesterol, mmol/l	2.5 (2.0-3.0)	2.1 (1.6-2.7)
Triacylglycerol, mmol/l	1.6 (1.2-2.2)	2.0 (1.4-2.7)

Data are displayed as median (IQR), except where otherwise indicated.

SNF analysis revealed that patients from both GoDARTS and DCS cohorts could be separated into two clusters based on their circulating plasma lipidomics and proteomics data (Figure 1A). The significance of the clusters (from now on referred to as subgroups) was validated by bootstrapping (n=1000 iterations) against simulated data (Supplementary Figure 2). We also performed the same clustering analysis using single -omics datasets for comparison and showed that the clustering results from the combined (proteomics +lipidomics) dataset are improved compared to single -omics results (Supplementary Figure 1). Subgroup 1 comprised 46.5% and 40.7%, respectively, of the cohort patients while subgroup 2 comprised 53.5% and 59.3%, respectively. The similarity between patient subgroups in DCS and GODARTS cohorts is represented as a network where the nodes represent patients and the connections between them (edges) represent the similarity between them (Figure 1B). Node connectivity was measured by betweenness centrality, and is reflected by the size of the nodes in the figure. Patients are coloured according to their subgroup labels showing that patient subgroups appear separated from each other in the network. The nodes with high betweenness are mostly positioned in the centre of the network, indicating similarity of these nodes to both subgroups.

To investigate whether similar data-driven separation of patients could be observed using an independent method, we used an unsupervised data fusion approach, *ComDim (CCSWA or Common Dimensions)*, to project patient similarity based on combined lipidomics and proteomics data into two dimensional space (23, 24). For both DCS and GoDARTS cohorts, this analysis showed clear separation of the two patient subgroups identified by SNF along dimension 2. Thus, using an independent method without any *a priori*, we were able to confirm that the subgroups reflect individuals with different underlying plasma lipidomics and proteomics profiles. Details of the features most associated with each common dimension are shown in Supplementary Table 1.

3.2 Clinical differences between subgroups

We next sought to determine whether the observed subgroups may differ in terms of their clinical characteristics. Homeostatic model assessment 2 (HOMA2) is a non-invasive, commonly used mathematical model for estimating beta-cell function (-B), insulin sensitivity (-S), and insulin resistance (-IR) (25). In DCS, subgroup 1 and subgroup 2 show significant differences in HOMA2 (p=0.0008;4.2e-11;1.1e-09, -B, -IR and -S) and C-peptide (p=3.7e-11) level. Patients in subgroup 1 showed higher HOMA2-B, HOMA2-IR and C-peptide levels compared to subgroup 2 (Figure 2A). Similar results were observed in GoDARTS (C-peptide p=2.5e-06) (Figure 2A). Since measurements in subjects from the GoDARTS cohort were performed in a non-fasting state, HOMA2 was not possible for this cohort. Furthermore, glycaemic deterioration, measured as the length of time to start insulin treatment (26), was higher for patients in subgroup 1 than

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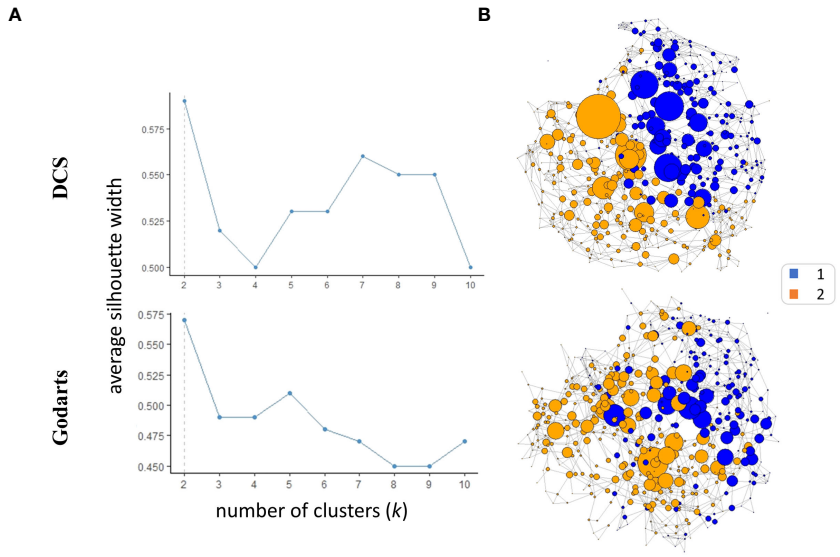

FIGURE 1
Similarity Network Fusion (SNF) identifies two multiomics clusters in independent cohorts. **(A)** Average silhouette width was calculated for SNF clusters identified in both cohorts with the number of clusters (*k*) ranging from 2 to 10. A high mean silhouette width for a cluster indicates that it is well separated from other clusters and is a measure of clustering quality. **(B)** The similarity networks were generated based on unweighted adjacency matrices. The unweighted adjacency matrices were derived from similarity matrices. In the context of similarity matrices, values exceeding the 97.5th percentile threshold were assigned a value of 1, while values below this threshold were assigned a value of 0.The nodes represent the patients where the size represents the node *betweenness centrality*. Nodes were coloured based on their cluster assignments.

subgroup 2 (Hazard Ratio=0.56; 0.73, DCS; GoDARTS, Figure 2B) in each cohort. In order to see how the subgroups compared to previously reported clinically derived T2D clusters in DCS and GoDARTS (5, 13), we assigned each patient to the relevant cluster label and calculated the significance of patient overlap with each molecular subgroup (Supplementary Table 2; Supplementary Figure 4). In DCS, a small but significant overlap was observed between subgroup 1 and severe insulin-resistant diabetes (SIRD) in DCS (intersect = 91; p-value = 1.13E-6) and mild obesity-related diabetes (MOD) (intersect = 67, p-value = 1.6E-03); and between subgroup 2 and mild diabetes with high HDL-cholesterol (MDH) (intersect = 100; p-value = 7.1E-09). Although less pronounced, a similar trend could also be observed in GoDARTS between subgroup 1 and SIRD (intersect = 51; p-value = 0.085), MOD (intersect = 61; p-value = 0.007) and between subgroup2 and MDH (intersect = 85; p-value = 0.003).

3.3 Patient subgroups display distinct molecular features

We next investigated the subgroups' molecular profiles using logistic regression models adjusting for sex, BMI and age. In DCS, 123 proteins and 137 lipids showed significant differences between subgroups. In GoDARTS, 130 proteins and 149 lipids showed significant differences between subgroups (Figure 3). 50 significant common peptides and 109 significant common lipids are shared between the DCS and GoDARTS cohorts.

A hierarchical clustering of the multi-dimensional subgroup-associated features is shown as a heatmap and box plot in DCS and GoDARTS (Figure 3; Supplementary Figure 5). In DCS and GoDARTS, several discriminative features can be observed. For lipids, triacylglycerol (TAG), Diacylglycerol (DAG) and Phosphatidylcholine (PC) show marked differences between clusters in both cohorts.

Patients in subgroup 1 also showed high levels of pro-inflammatory proteins such as interleukin 18 receptor 1 (IL18-R1), interleukin 1 receptor 1 (IL1-R1) and interleukin 19. Moreover, patients in subgroup 1 also had higher levels of proteins related to cellular growth such as growth factor receptor binding protein 2, growth hormone receptor, as well as glucose and fatty acid metabolism-related proteins such as glucose 6 phosphate isomerase (GPI) and 3 hydroxyacyl CoA dehydrogenase (HADH). In contrast, patients in subgroup 2 showed higher levels of immune regulatory proteins such as coactosin-like protein, COD29 antigen and lymphocyte antigen. Furthermore, tyrosine protein kinases family, -Lyn, Fyn and -BTK are also more abundant in subgroup 2 than subgroup 1. Interestingly, several blood coagulation and platelet proteins such as complement component C9, E-selectin and platelet factor 4 showed significant differences between subgroups in both cohorts (Figure 3; Supplementary Figure 5).

4 Discussion

Building on our recent study, which primarily centred on testing individual biomarkers for association with glycaemic deterioration (4), in the present report, we employed an alternative multimodal, multivariate strategy to capture the complexity of molecular interactions associated with T2D. Using

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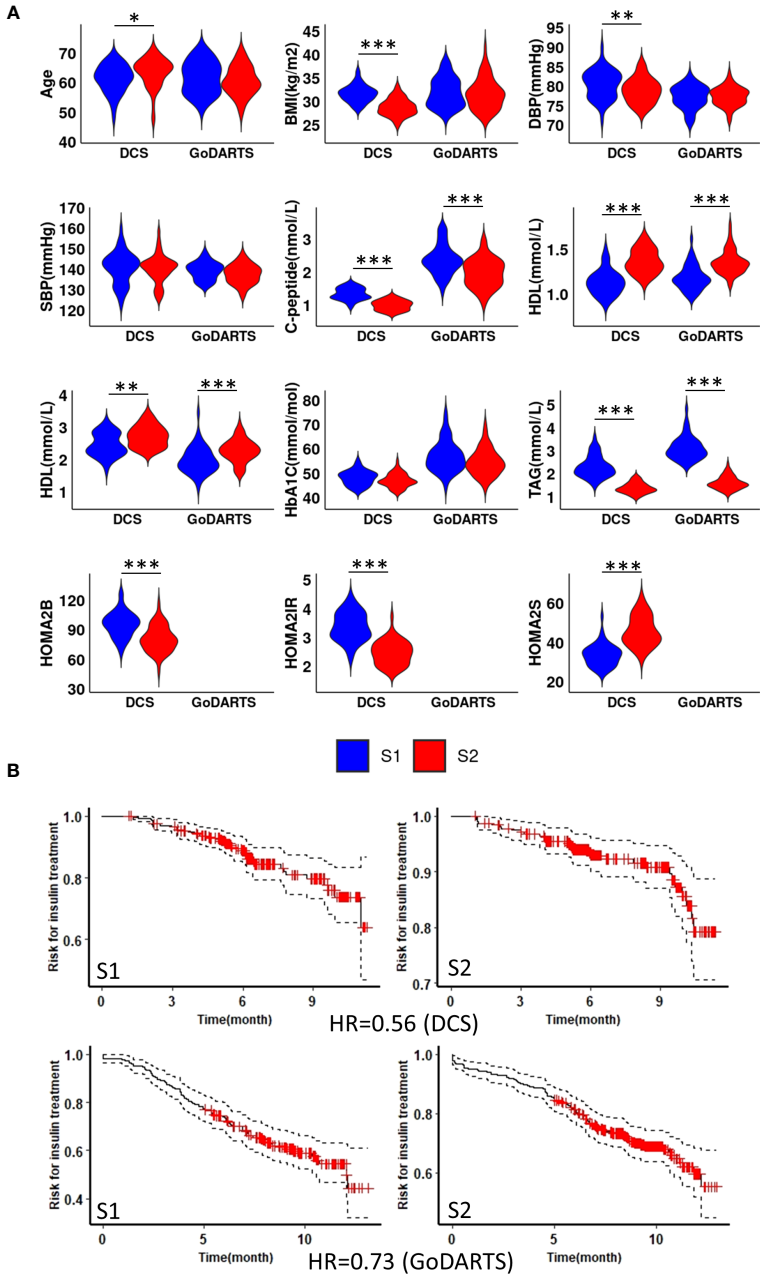

FIGURE 2
Subgroup 1 shows clinical characteristics of more severe disease and faster glycaemic deterioration. **(A)** Distributions of nine clinical measurements at baseline in both DCS and GoDARTS cohorts for each subgroup. **(B)** Cox proportional hazards models with the time required for insulin treatment between subgroup 1 and subgroup 2 in both DCS (upper) and GoDARTS (lower). The hazard ratio for the second group relative to the first group is shown. DBP, Diastolic Blood Pressure; SBP, Systolic Blood Pressure; BMI, Body Mass Index; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; TAG, Triglycerides; HbA1C, Haemoglobin A1C; HOMA2, Homeostasis Model Assessment 2; DCS, Hoorn Diabetes Care System; GoDARTS, Genetics of Diabetes Audit and Research in Tayside Scotland; S1, Multi-omics subgroup 1; S2, Multi-omics subgroup 2. *P-value<=0.05. **P-value<=0.01. ***P-value<=0.001.

an unsupervised network-based data fusion method, SNF, we were able to group T2D individuals with similar multi-omics profiles into two robust subgroups from two independent European cohorts. In the DCS cohort, patients assigned to subgroup 1 and 2 showed clear differences in insulin resistance and beta-cell function as measured by HOMA2-B, -IR, C-peptide, and triglyceride levels, with similar results obtained in the GoDARTS cohort (27). Thus subgroup 1 appears to represent individuals with both increased insulin resistance *and* islet beta cell function at diagnosis.

We also provide evidence from Cox proportional hazards modelling in both cohorts that patients assigned to the different subgroups show altered rates of disease progression as defined by time of diagnosis to requirement for insulin treatment. We note that in the current and prior studies (4), we ascertained the time to

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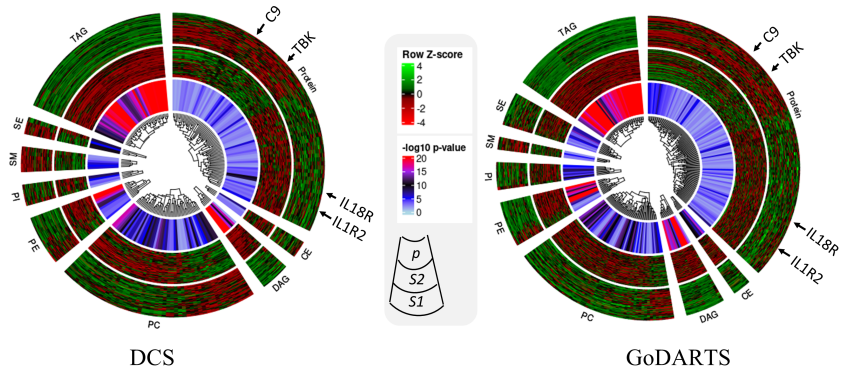

FIGURE 3
Circular heatmap showing similarities between multi-omics signatures in DCS and GoDARTS. Concentrations of each biomarker (logistic regression; $p<0.05$) were first converted to log(concentration) and then normalised (Row Z-score). Each vertical line in the circular heatmap represents one biomarker and each circle represents one patient. The heatmap was grouped based on first type of molecule, followed by hierarchical clustering. The different layers in the circle are (from outer to inner circle): subgroup1 molecular expression heatmap; subgroup 2 molecular expression heatmap; logistic regression p-value. For more details of each individual molecule profile, please refer to Supplementary Figure 5. CE, Ceramide; DAG, Diacylglycerols; PC, Phosphatidylcholines; PE, Phosphatidylethanolamine; PI, Phosphatidylinositols; SM, Sphingomyelins; SE, Sterol Esters; TAG, Triacylglycerols; DCS, Hoorn Diabetes Care System; GoDARTS, Genetics of Diabetes Audit and Research in Tayside Scotland; S1, Multi-omics subgroup 1; S2, Multi-omics subgroup 2.

insulin initiation based on two criteria: either the duration before an individual commenced insulin treatment post-diagnosis, or when a consistently elevated HbA1c levels was observed despite the use of multiple (>2) non-insulin medications. This is an accepted approach to effectively quantify glycaemic deterioration (26)

Amongst the lipids showing differences between the two subgroups, TAG, DAG and PC showed significantly higher levels in subgroup 1 than in subgroup 2, which potentially reflects an association between circulating fatty acids and insulin resistance (28, 29). In contrast, Sphingomyelin (SM) species were present at higher levels in subgroup 2 compared to subgroup 1. Similar results were also observed in our previous study (4) where decreased levels of sphingomyelin SM 42:2;2 was a predictor of more rapid glycaemic deterioration.

The proteins associated with the present study subgroups fell into a number of different categories, notably immune regulatory related proteins; metabolic enzymes e.g., Alcohol dehydrogenase; hormones and growth factors; signalling proteins; and blood coagulation factors e.g., E-selectin. Notably, several novel T2D biomarkers, which were identified in our previous research as associated with differing rates of T2D progression (4) were significantly altered between subgroups in the present report, suggesting that they form part of a molecular signature associated with disease severity.

Previously, we detected immune regulation proteins IL-18R1, CRELD1 and coactosin-like-protein, to be associated with T2D progression across multiple independent studies (4). Of interest the levels of these proteins are also significantly different between the two subgroups identified in the present study. Moreover, patients in subgroup 1 also showed higher expression levels of other immune-related proteins e.g. CC motif chemokine 15/16/25, ferritin, IL-1R2 and IL-19 and lower level of COD29 and coactosin-like protein (Supplementary Figure 5) highlighting the value of using an unsupervised multivariate approach for detecting disease related signatures which may be missed by more conventional supervised approaches.

The goal of the present study was to use plasma lipidomics and proteomics data to uncover -omics signatures related to disease severity. Thus, the two subgroups we identify should be primarily seen as tools to help investigate molecular signatures underlying disease rather than as a means to cluster or stratify patients. Nevertheless, it is meaningful to compare these subgroups with previously identified data driven clusters (5) since these latter clusters have been validated and extensively analysed in the same two cohorts (8, 13). The comparison showed small but significant overlap between subgroup1 and SIRD and MOD, and between subgroup2 and MDH indicating that although there are some similarities, the multi-omics-based subgroups do not closely correspond to any of these previously identified clusters. This underscores the fact that clustering will give different results depending on what data is used and is best employed to observe tendencies, trends or patterns in underlying data rather than for strict patient stratification.

During our analysis, we also tested the subgroups for enrichment of ~400 known genetic loci associated with T2D (30) since this could provide evidence for causal association to disease. However, we found no significant enrichment of any of the T2D variants within the subgroups, possibly reflecting lack of power to detect such associations. The data used in this study, although limited in terms of absolute number of patients, is substantial considering the cost of generating the proteomics data alone (>1000USD/sample). Nevertheless, although high cost precludes performing this type of analysis on large populations or routinely in the clinic, it would be beneficial in future studies to profile large numbers of individuals with T2D with selected panels of protein/lipid biomarkers similar to the ones presented here. Measuring biomarkers over time is especially relevant as T2D is dynamic and underlying molecular signatures may change as the disease

progresses. Indeed, biomarkers such as TAG and DAG can be subject to changes reflecting an individual's lifestyle and/or medication. These aspects will need to be assessed in future prospective studies.

5 Limitations of the study

The subgroups in our study were derived primarily from European patients. Consequently, the applicability of these results to other ethnic groups is uncertain. In both DCS and GoDARTS, patients were not necessarily recruited after diagnosis. This heterogeneity in the disease duration could potentially have an effect on the blood measurements obtained. Moreover, as mentioned above, the absence of C-peptide measurements in the fasted state precluded HOMA assessments in the GoDARTS cohort. The effect of fasting or non-fasting state on the multi-omics profiling in the current study is still not fully clear and needs further investigation (31, 32).

6 Conclusions

We demonstrate that an unsupervised, "bottom-up" multi-omics approach can segregate T2D patients into 2 subgroups capturing differences in insulin resistance and glycaemic deterioration. Several classes of biomarkers, notably those involved in immune processes, were most strongly associated with these subgroups and future investigations will be necessary to establish their causal roles, if any, in disease progression.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding authors.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the patients/participants or patients/participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

SL: Formal analysis, Investigation, Methodology, Writing – original draft. ID: Methodology, Software, Data curation, Writing – review & editing. VT: Methodology, Writing – review & editing. CH: Methodology, Investigation, Writing – review & editing. DK: Methodology, Writing – review & editing. MH: Conceptualization, Resources, Writing – review & editing. JB: Conceptualization, Data curation, Writing – review & editing. LH: Conceptualization, Writing – review & editing. RS: Methodology, Writing – review & editing. LD: Data curation, Methodology, Writing – review & editing. MG: Methodology, Writing – review & editing. CK: Methodology, Writing – review & editing. FM: Methodology, Writing – review & editing. KS: Funding acquisition, Methodology, Supervision, Writing – review & editing. PE: Methodology, Writing – review & editing. EP: Funding acquisition, Supervision, Writing – review & editing. GR: Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing, Project administration, Writing – original draft. MI: Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing, Writing – original draft.

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Conflict of interest

GR has received grant funding from, and is a consultant for, Sun Pharmaceuticals Inc. KS is CEO of Lipotype. KS and CK are shareholders of Lipotype. MG is an employee of Lipotype. MH is an employee of Janssen Research & Development. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1350796/full#supplementary-material

References

1. Visualizing heterogeneity in type 2 diabetes phenotype, outcome and drug response. *Nat Med*. (2022) 28:909–10. doi: 10.1038/s41591-022-01791-6

2. Nair ATN, Wesolowska-Andersen A, Brorsson C, Rajendrakumar AL, Hapca S, Gan S, et al. Heterogeneity in phenotype, disease progression and drug response in type 2 diabetes. *Nat Med*. (2022) 28:982–8. doi: 10.1038/s41591-022-01790-7

3. Delhalle S, Bode SFN, Balling R, Ollert M, He FQ. A roadmap towards personalized immunology. *NPJ Syst Biol Appl*. (2018) 4:9. doi: 10.1038/s41540-017-0045-9

4. Slieker RC, Donnelly LA, Akalestou E, Lopez-Noriega L, Melhem R, Güneş A, et al. Identification of biomarkers for glycaemic deterioration in type 2 diabetes. *Nat Commun*. (2023) 14:2533. doi: 10.1038/s41467-023-38148-7

5. Ahlqvist E, Storm P, Käräjämäki A, Martinell M, Dorkhan M, Carlsson A, et al. Novel subgroups of adult-onset diabetes and their association with outcomes: a data-driven cluster analysis of six variables. *Lancet Diabetes Endocrinol*. (2018) 6:361–9. doi: 10.1016/S2213-8587(18)30051-2

6. McCarthy MI. Painting a new picture of personalised medicine for diabetes. *Diabetologia*. (2017) 60:793–9. doi: 10.1007/s00125-017-4210-x

7. Florez JC, Pearson ER. A roadmap to achieve pharmacological precision medicine in diabetes. *Diabetologia*. (2022) 65:1830–38. doi: 10.1007/s00125-022-05732-3

8. Slieker RC, Donnelly LA, Fitipaldi H, Bouland GA, Giordano GN, Åkerlund M, et al. Distinct molecular signatures of clinical clusters in people with type 2 diabetes: an IMI-RHAPSODY study. *Diabetes*. (2021) 70:2683–93. doi: 10.2337/db20-1281

9. Wolfson M, Wallace SE, Masca N, Rowe G, Sheehan NA, Ferretti V, et al. DataSHIELD: resolving a conflict in contemporary bioscience–performing a pooled analysis of individual-level data without sharing the data. *Int J Epidemiol*. (2010) 39:1372–82. doi: 10.1093/ije/dyq111

10. Wang B, Mezlini AM, Demir F, Fiume M, Tu Z, Brudno M, et al. Similarity network fusion for aggregating data types on a genomic scale. *Nat Methods*. (2014) 11:333–7. doi: 10.1038/nmeth.2810

11. van der Heijden AA, Rauh SP, Dekker JM, Beulens JW, Elders P, 't Hart LM, et al. The Hoorn Diabetes Care System (DCS) cohort. A prospective cohort of persons with type 2 diabetes treated in primary care in the Netherlands. *BMJ Open*. (2017) 7: e015599.

12. Hébert HL, Shepherd B, Milburn K, Veluchamy A, Meng W, Carr F, et al. Cohort profile: genetics of diabetes audit and research in tayside scotland (GoDARTS). *Int J Epidemiol*. (2018) 47:380–1j. doi: 10.1093/ije/dyx140

13. Slieker RC, Donnelly LA, Fitipaldi H, Bouland GA, Giordano GN, Åkerlund M, et al. Replication and cross-validation of type 2 diabetes subtypes based on clinical variables: an IMI-RHAPSODY study. *Diabetologia*. (2021) 64:1982–9. doi: 10.1007/s00125-021-05490-8

14. Doiron D, Burton P, Marcon Y, Gaye A, Wolffenbuttel BHR, Perola M, et al. Data harmonization and federated analysis of population-based studies: the BioSHaRE project. *Emerg Themes Epidemiol*. (2013) 10:12. doi: 10.1186/1742-7622-10-12

15. Doiron D, Marcon Y, Fortier I, Burton P, Ferretti V. Software Application Profile: Opal and Mica: open-source software solutions for epidemiological data management, harmonization and dissemination. *Int J Epidemiol*. (2017) 46:1372–8. doi: 10.1093/ije/dyx180

16. Gaye A, Marcon Y, Isaeva J, LaFlamme P, Turner A, Jones EM, et al. DataSHIELD: taking the analysis to the data, not the data to the analysis. *Int J Epidemiol*. (2014) 43:1929–44. doi: 10.1093/ije/dyu188

17. Dragan I, Sparsø T, Kuznetsov D, Slieker R, Ibberson M. dsSwissKnife: An R package for federated data analysis. *bioRxiv*. (2020). doi: 10.1101/2020.11.17.386813

18. Dragan I. dsSwissKnifeClient: DataSHIELD Tools and Utilities - client side. R package version 0.2.0 (2021), GitHub repository. (2021). Available at: https://github.com/sib-swiss/dsSwissKnifeClient.git

19. Xu T, Le TD, Liu L, Su N, Wang R, Sun B, et al. CancerSubtypes: an R/Bioconductor package for molecular cancer subtype identification, validation and visualization. *Bioinformatics*. (2017) 33:3131–3. doi: 10.1093/bioinformatics/btx378

20. Kembel SW, Cowan PD, Helmus MR, Cornwell WK, Morlon H, Ackerly DD, et al. Picante: R tools for integrating phylogenies and ecology. *Bioinformatics*. (2010) 26:1463–4. doi: 10.1093/bioinformatics/btq166

21. Csardi G, Nepusz T. The igraph software package for complex network research. *InterJournal Complex Syst*. (2006) 1695.

22. Warnes G, Bolker B, Bonebakker L, Gentleman R, Liaw W, Lumley T, et al. gplots: Various R Programming Tools for Plotting Data, GitHub repository. Available at: https://github.com/talgalili/gplots.git

23. Qannari EM, Wakeling I, Courcoux P, MacFie HJH. Defining the underlying sensory dimensions. *Food Qual Prefer*. (2000) 11:151–4. doi: 10.1016/S0950-3293(99)00069-5

24. Qannari EM, Courcoux P, Vigneau E. 'Common components and specific weights analysis performed on preference data'. *Food Quality and Preference*. (2001) 12(5-7):365–8. doi: 10.1016/S0950-3293(01)00026-X

25. Levy JC, Matthews DR, Hermans MP. Correct homeostasis model assessment (HOMA) evaluation uses the computer program. *Diabetes Care*. (1998) 21:2191–2. doi: 10.2337/diacare.21.12.2191

26. Zhou K, Donnelly LA, Morris AD, Franks PW, Jennison C, Palmer CN, et al. Clinical and genetic determinants of progression of type 2 diabetes: a DIRECT study. *Diabetes Care*. (2014) 37:718–24. doi: 10.2337/dc13-1995

27. Jiang L, Xiao Y, Ding Y, Tang J, Guo F. Discovering cancer subtypes via an accurate fusion strategy on multiple profile data. *Front Genet*. (2019) 10:20. doi: 10.3389/fgene.2019.00020

28. Glueck CJ, Khan NA, Umar M, Uppal MS, Ahmed W, Morrison JA, et al. Insulin resistance and triglycerides. *J Investig Med*. (2009) 57:874–81. doi: 10.2310/JIM.0b013e3181bca9d2

29. Boden G. Free fatty acids, insulin resistance, and type 2 diabetes mellitus. *Proc Assoc Am Physicians*. (1999) 111:241–48. doi: 10.1046/j.1525-1381.1999.99220.x

30. Mahajan A, Taliun D, Thurner M, Robertson NR, Torres JM, Rayner NW, et al. Fine-mapping type 2 diabetes loci to single-variant resolution using high-density imputation and islet-specific epigenome maps. *Nat Genet*. (2018) 50:1505–13. doi: 10.1038/s41588-018-0241-6

31. Driver SL, Martin SS, Gluckman TJ, Clary JM, Blumenthal RS, Stone NJ. Fasting or nonfasting lipid measurements: it depends on the question. *J Am Coll Cardiol*. (2016) 67:1227–34. doi: 10.1016/j.jacc.2015.12.047

32. Liu MM, Peng J, Cao YX, Guo YL, Wu NQ, Zhu CG, et al. The difference between fasting and non-fasting lipid measurements is not related to statin treatment. *Ann Transl Med*. (2021) 9:386. doi: 10.21037/atm-20-3962

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Proinflammatory cytokines suppress nonsense-mediated RNA decay to impair regulated transcript isoform processing in pancreatic β cells

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Introduction: Proinflammatory cytokines are implicated in pancreatic ß cell failure in type 1 and type 2 diabetes and are known to stimulate alternative RNA splicing and the expression of nonsense-mediated RNA decay (NMD) components. Here, we investigate whether cytokines regulate NMD activity and identify transcript isoforms targeted in ß cells.

Methods: A luciferase-based NMD reporter transiently expressed in rat INS1(832/13), human-derived EndoC-ßH3, or dispersed human islet cells is used to examine the effect of proinflammatory cytokines (Cyt) on NMD activity. The gain- or loss-of-function of two key NMD components, UPF3B and UPF2, is used to reveal the effect of cytokines on cell viability and function. RNA-sequencing and siRNA-mediated silencing are deployed using standard techniques.

Results: Cyt attenuate NMD activity in insulin-producing cell lines and primary human ß cells. These effects are found to involve ER stress and are associated with the downregulation of UPF3B. Increases or decreases in NMD activity achieved by UPF3B overexpression (OE) or UPF2 silencing raise or lower Cyt-induced cell death, respectively, in EndoC-ßH3 cells and are associated with decreased or increased insulin content, respectively. No effects of these manipulations are observed on glucose-stimulated insulin secretion. Transcriptomic analysis reveals that Cyt increases alternative splicing (AS)-induced exon skipping in the transcript isoforms, and this is potentiated by

UPF2 silencing. Gene enrichment analysis identifies transcripts regulated by UPF2 silencing whose proteins are localized and/or functional in the extracellular matrix (ECM), including the serine protease inhibitor SERPINA1/α-1-antitrypsin, whose silencing sensitizes ß-cells to Cyt cytotoxicity. Cytokines suppress NMD activity via UPR signaling, potentially serving as a protective response against Cyt-induced NMD component expression.

Conclusion: Our findings highlight the central importance of RNA turnover in ß cell responses to inflammatory stress.

KEYWORDS

β-cells, insulin secretion, transcript, nonsense-mediated decay, RNA decay, RNA processing

Introduction

Inflammatory and glucolipotoxic (GLT) stress causing β-cell failure and destruction *in vitro* differentially regulates hundreds of β-cell transcripts (1, 2). The upregulation of splicing factors and of proteins involved in pre-mRNA processing gives rise to alternative splicing (AS) events, which in turn deregulates the balance and turnover of transcript isoforms (3). Interestingly, most human mRNAs exhibit alternative splicing, but not all alternatively spliced transcripts are translated into functional proteins and are therefore targeted for degradation via the RNA decay pathways.

In addition to regulating the expression of normal transcripts, the human nonsense-mediated RNA decay (NMD) machinery functions to eliminate premature termination codon (PTC)-containing mRNAs, as reviewed extensively (4). Alternatively, spliced mRNA species and translation of dominant transcript isoforms vary in a cell-specific manner and depend on the capacity of cells to cope with damaged transcripts (5–7). A substantial number (i.e., around 35%, depending on tissue and physiological conditions) of alternatively spliced variants contain a PTC (4, 8, 9). Approximately 35% of the cytokine-regulated transcripts in human islets undergo alternative splicing (6), and Cyt profoundly upregulates NMD in rat and human insulin-producing cell lines and primary β cells, likely to handle the NMD load inferred by PTC-containing splice variants (4, 10, 11).

However, in addition to canonical NMD, in which all key NMD components function on target transcripts, a second branch of NMD is (in)dependently regulated in an autoregulatory feedback loop by its key factors, including UPF2 and UPF3 in a cell type-specific manner, as reviewed previously (4, 10).

In a previous study (11), we profiled the expressional level of NMD components and their regulation by cytokines and GLT in insulin-producing cells, but the NMD activity and its consequences for the β-cell transcriptome remained to be investigated. Here, using a luciferase-based NMD activity reporter, gain-/loss-of-function, and RNA-sequencing analyses in rodent and human β-cell systems, we measured NMD activity and explored its consequences for the function and viability of pancreatic β cells under normal conditions and inflammatory stress.

Materials and methods

Cell culture, human islet dispersion, and treatment

INS1(832/13) (12), EndoC-βH3 (13), or dispersed human islet cells were cultured and manipulated according to the protocols and procedures described in the Supplementary Methods.

Luciferase-based NMD activity assay

One million cells were cotransfected with 650 ng of plasmid encoding either human *Haemoglobin-β* (*HBB*) wildtype (WT or PTC^-) or with a PTC-containing mutation (NS39 or PTC^+) fused with *Renilla* (*RLuc*), in brief, named PTC^- and PTC^+, respectively. *Firefly* (*FLuc*) plasmid (14) was used as a transfection efficiency reference. *Renilla* and *Firefly* luminescence were measured by Dual-Luciferase Reporter Assay (Promega, Hampshire, England) (Supplementary Methods). *RLuc* signals were normalised to the *FLuc* control in both HBB NS39, in the following named HBB (PTC^+), and HBBWT, in the following named HBB (PTC^-), and NMD activity was calculated by dividing the *RLuc/FLuc*-HBB (PTC^-) by the *RLuc/FLuc*-HBB (PTC^+) (Supplementary Figure S1A) (14). Experiments where the control construct *RLuc/FLuc*-HBB(PTC^-) was affected by cytokines were excluded, so that the resulting NMD activity only denotes the PTC-containing HBB (PTC^+). The transfection efficiency was tested twice and resulted in an average of 80% in INS1 and EndoC-βH3 cells, as measured by FACS analysis of cells transfected with a GFP-expressing plasmid (Supplementary Figures S1B, C).

Functional analysis of UPF3A/B overexpression

One million INS1(832/13) or EndoC-βH3 cells were transfected with 650 ng of plasmids encoding UPF3A, UPF3B, or UPF3BΔ42 (15), then simultaneously with NMD activity reporter plasmids (as above, 650 ng/million cells), recounted and seeded for Western blotting, glucose-stimulated insulin secretion (GSIS), viability, apoptosis (detailed below), and NMD activity assays in relevant plates and preincubated for 48 h before treatment with cytokines as explained in the Supplementary Methods.

Lentiviral shRNA gene knockdown

GPIZ lentiviral shRNA particles directed against *UPF2*, *Upf3A*, or *Upf3B*, and a nonsilencing shRNA (NS) as a negative control were produced using the Trans-Lentiviral shRNA Packaging System in HEK293 cells (Horizon, Cambridge, England) according to the manufacturer's protocol (Supplementary Methods).

Apoptosis and cell viability assays

Apoptosis assays were performed in duplicate by detection of caspase-3 activity using a fluorometric (μM AMC) (or/colourimetric [μM PNA/min/mL] unless stated) assay kit (Cat No. APPA015-1KT/CASP3C-1KT, Sigma, London, England) according to the manufacturer's protocols. Cell viability was measured by the Alamarblue assay (Cat No. DAL1025, LifeTechnologies, Renfrew, England) as previously described (11).

Library preparation, RNA-sequencing, and data analysis

Using TRIZOL, 33 independent biological replicates of total RNA from the NS control and/or UPF2 KD EndoC-βH3 cells exposed to cytokines, GLT, or PBS (i.e., $N = 6$ of each PBS-/or cytokine-exposed NS control and UPF2 KD, and $N = 4/N = 5$ of GLT-exposed NS control/UPF2 KD, respectively) were extracted, treated with DNase, and precipitated with isopropanol (Supplementary Methods). Following the manufacturer's recommendations, 1 μg of total RNA/per isolate was used as input for the generation of sequencing libraries using NEBNext®Ultra-TM RNA Library-Prep (Cat No. E7770, NEB, Ipswich, MA, USA) (Supplementary Methods). The RNA-seq raw data underwent quality control and were mapped to the human reference genome (16) and analysed using the bioinformatic pipeline described in the Supplementary Methods.

cDNA synthesis and RT-qPCR

Purified total RNA (500 ng) was used for cDNA synthesis with the SuperScript™ (Cat No. 11904018, LifeTechnologies). Real-time reverse transcriptase-quantitative PCR (RT-qPCR) was performed on 12 ng cDNA with SybrGreen PCR Master Mix (LifeTechnologies) and specific primers (Supplementary Table S2) and run in an ABI Real-Time PCR Machine (Applied Biosystems, ThermoFisher Scientific, Oxford, England). The raw data was analysed through $-\Delta Ct$ as described in the Supplementary Methods.

Western blotting

Western blotting was performed using antibodies against alpha-tubulin (1:2,000) (Cat No. T5168, Sigma), UPF2 (1:1,000) (Cat No. PA5-77128, LifeTechnologies), UPF3A (1:1,000) (Cat No. PA5-41904, LifeTechnologies), UPF3B (1:1,000) (Cat No. PB9843, Boster-Bio, Pleasanton, CA, USA) and α-1-antitrypsin (1:1,000) (Cat No. TA500375, LifeTechnologies) as described in the Supplementary Methods (17).

Glucose-stimulated insulin secretion

In 12-well plates (Cat No. 150200, Nunc, Buckingham, England), 300,000 INS1(832/13) or EndoC-βH3 cells were cultured and preincubated for 2 days. GSIS was carried out using Krebs–Ringer buffer containing 2 mM or 17 mM glucose, as described (11, 18).

Insulin assay

Insulin concentration (ng/mL or pM) was measured using a rat insulin ultrasensitive ELISA kit (Cat No. 62IN2PEG, Cisbio, Cambridge, England) or human insulin ELISA Kit (Cat No. 90095, CrystalChem, IL, USA), respectively, according to the manufacturer's protocol.

Statistical analysis

Data are presented as means ± SEM. Statistical analysis was carried out on raw data in cases where figures gave normalised data. Group comparisons were carried out by two- or one-way ANOVA as appropriate. Significant ANOVAs were followed by a *post-hoc* paired Student's *t*-test with Bonferroni correction using GraphPad Prism 6.0 (La Jolla, CA, USA). A paired *t*-test was chosen to normalise for interpassage variability in outcome parameters. Since the experimental conditions did not allow sequential sampling from the same cell culture, parallel control and interventional plate wells were considered to be paired observations and analysed accordingly statistically. If the *post-hoc* paired *t*-test did not reveal a carrying statistical difference by ANOVA, individual paired *t*-tests were performed and corrected for multiple comparisons. Bonferroni-corrected p-values ≤ 0.05 were considered significant and ≤ 0.10 a trend.

Results

Cytokines suppress NMD activity in β cells

We previously reported that cytokines and glucolipotoxicity differentially up- or downregulate NMD component transcripts in pancreatic β cells (11). However, whether this regulation leads to increased NMD *activity* remains to be elucidated. Here, we used a luciferase-based NMD reporter (Supplementary Figure S1A) (14) to examine NMD activity in rat INS1(832/13), human insulin-producing EndoC-βH3 cells, and primary human islets. Luciferase activity analysis showed that cytokines (Cyt; 150 pg/mL IL-1β + 0.1 ng/mL IFN-γ + 0.1 ng/mL TNF-α) significantly suppressed NMD activity by nearly 50% after 18 h, but not 6 h, of exposure in INS1(832/13) cells [Supplementary Figures S2A (i, iii)].

We then tested the effects of cytokines on EndoC-βH3 cells and dispersed human islet cells. Cytokines [2.5 ng/mL IL-1β + 10 ng/mL TNF-α + 10 ng/mL IFN-γ, chosen from dose–response experiment shown in Supplementary Figure S2B (i)] attenuated NMD activity by 30% ($p = 0.009$, $n = 6$) and 40% ($p = 0.0006$, $n = 6$) after 18 h of exposure to EndoC-βH3 cells (Figure 1Ai) and dispersed human islet cells (Figure 1B), respectively. Cyt increased the luciferase signal (*RLuc/FLuc*) from the HBB(PTC$^-$) (Supplementary Figures S2B (ii), C) but not from the HBB(PTC$^-$) [Supplementary Figures 2B (v), C (ii)] in both cell models, confirming that the NMD substrate HBB(PTC$^+$) was restored due to NMD activity attenuation by Cyt.

We next examined whether cytokine-mediated suppression of NMD was consistent with an accumulation of HBB(PTC$^+$) *transcripts*. For this, we used a forward and reverse primer set to amplify the *Renilla* gene and the junction of exons 1 and 2 (i.e., ensuring amplification of mature transcripts only), respectively. RT-qPCR analysis demonstrated that cytokines caused significant upregulation of HBB(PTC$^+$) but not HBB(PTC$^-$) mRNA levels, rendering a significant reduction of the relative PTC$^-$/PTC$^+$ mRNA levels in INS1(832/13) ($p = 0.008$) [Supplementary Figure S2A (v, vi)] and EndoC-βH3 ($p = 0.001$) cells [Figure 1Aii; Supplementary Figure 2B (iv)], which verified the suppressive effect of cytokines on NMD activity.

We also examined the effect of 25 mM glucose or GLT conditions on NMD activity. Unfortunately, these conditions significantly affected the luciferase signal (*RLuc/FLuc*) from the HBB(PTC$^-$) control, preventing further study of the effects of metabolic stressors on NMD activity.

Taken together, these results show that cytokines suppress the activity of the NMD in a range of insulin-secreting cell types.

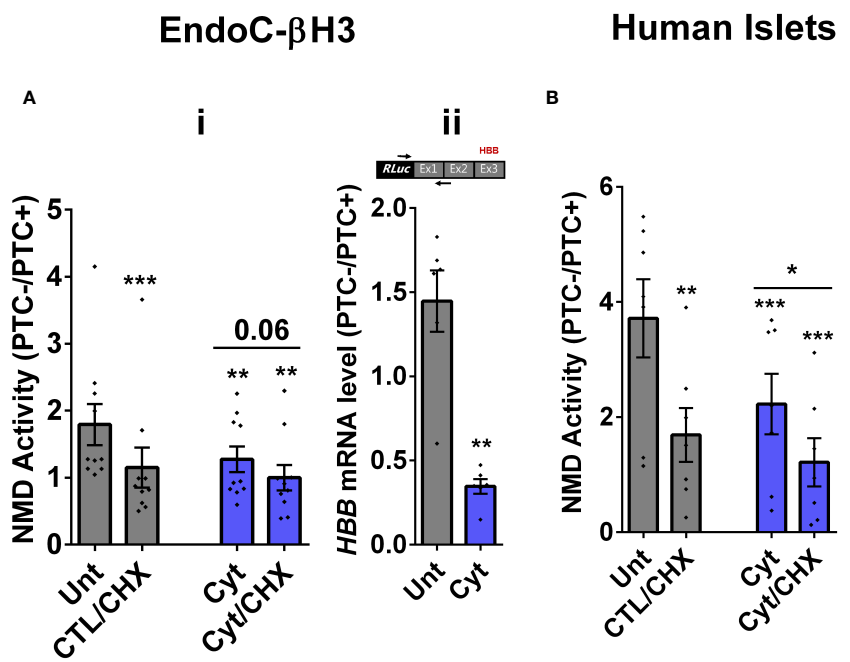

FIGURE 1
Cytokines suppress NMD activity in β cells. **(A, B)** EndoC-βH3 cells **(A)** and dispersed human islet cells **(B)** were cotransfected with *Renilla*-HBB (WTnamed PTC$^-$) and or *Renilla*-HBB (NS39 (named PTC$^+$)) and the *Firefly* plasmids and exposed to cytokine combination and or PBS as untreated (Unt) simultaneously with or without cycloheximide (CHX) as a positive control for inhibited NMD activity. Luciferase activity was measured in the lysate of the transfected EndoC-βH3 cells **(A** (i))and dispersed human islet cells **(B)** exposed to cytokine combination (Cyt; 3 ng/mL IL-1β + 10 ng/mL IFN-γ + 10 ng/mL TNF-α) for 18 (h) **(A** (ii)) mRNA level of *Renilla-HBB*-fused gene and the *Firefly* gene in the transfected EndoC-βH3 cells was quantified by RT-qPCR using specific primers extending the junction of exons 1 and 2 of the *HBB* gene and the *Renilla* gene, or only the *Firefly* gene, and normalised to actin and tubulin, respectively. The data are means ± SEM of $N = 6$. The symbol "*" indicates the Bonferroni-corrected paired *t*-test values of treated versus untreated (Unt) **(A, B)** or otherwise, cytokines (Cyt) that are designated by a line on top of the bars **(A, B)**: *≤ 0.05; **≤ 0.01; ***≤ 0.001; ****≤ 0.0001. ns, nonsignificant; HBB, *haemoglobin-β*; PTC, premature termination codon; RLuc, *Renilla* luciferase; Ex, exon.

Cytokine-induced suppression of NMD activity in β cells is ER stress-dependent

Whereas NMD degrades unfolded protein response (UPR)-induced transcripts in compensated ER stress, NMD is suppressed in response to pronounced endoplasmic reticulum (ER) stress to allow a full-blown UPR (19, 20). Cytokines induce a robust ER stress in pancreatic β cells, largely via nuclear factor-κB (NF-κB) activation and production of nitroxidative species that inhibit the smooth endoplasmic reticulum Ca^{2+} ATPase (SERCA) 2B pump, leading to ER calcium depletion (11, 17). We have previously shown that chemical inhibition of inducible nitric oxide synthase (iNOS) alleviated ER stress and normalised cytokine-mediated regulation of NMD components in INS1 cells (11). Therefore, we asked if cytokine-mediated reduction of NMD activity was dependent on an ER stress response in β cells. We first demonstrate that thapsigargin (TG), a noncompetitive inhibitor of SERCA (21) and ER stress inducer (22), inhibited NMD activity by 50% in EndoC-βH3 cells, as measured by luciferase assay [Supplementary Figures S3A (i, ii)]. Compared to untreated EndoC-βH3 cells (Unt), cytokines significantly augmented the increase in mRNA levels encoding the ER stress markers BiP, Xbp1, and Chop (FDR < 0.05) measured by RNA-sequencing analysis (Figure 2Ai) and later verified by RT-qPCR examination (Figure 2Aii–vi). Finally, compared with Unt, cytokines significantly decreased the NMD activity by 30%, and this effect was counteracted by the protein kinase R-like endoplasmic reticulum kinase (PERK) phosphorylation inhibitor GSK157 (8 μM) and by the inositol-requiring enzyme 1 (IRE1α) endoribonuclease inhibitor 4μ8C (16 μM) in EndoC-βH3 cells [Figure 2Bi; Supplementary Figures S3B, C (i, ii)]. RT-qPCR analysis of the relative PTC^-/PTC^+ mRNA levels in EndoC-βH3 cells confirmed the NMD activity data [Figure 2Bii, Supplementary Figure S3C (iii)].

Taken together, these results demonstrate that inhibition of UPR antagonises the cytokine-mediated reduction of NMD activity in EndoC-βH3, indicating that cytokine-mediated inhibition of NMD activity is UPR-dependent.

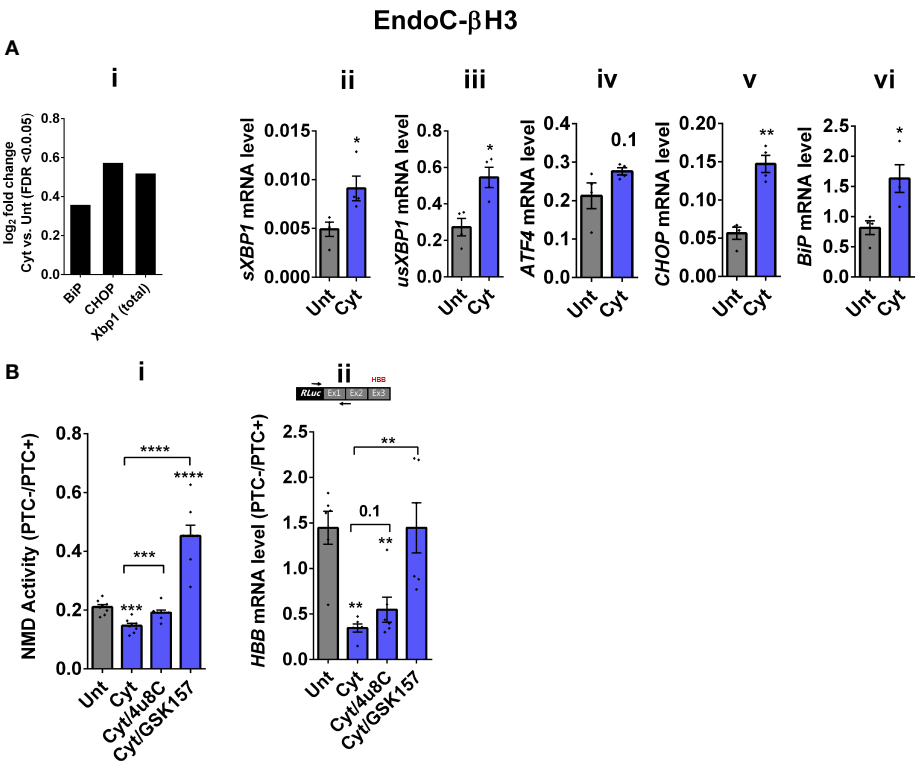

FIGURE 2
Cytokine-induced suppression of NMD activity in β cells is ER stress-dependent. **(A)** mRNA levels of ER stress markers in EndoC-βH3 cells exposed to cytokine combination (Cyt; 3 ng/mL IL-1β + 10 ng/mL IFN-γ + 10 ng/mL TNF-α) for 18 h were quantified by RNA sequencing (**A**(i)) with false discovery rate (FDR) < 0.05 presented as logarithmic fold change of the cytokine (Cyt) treatment versus control (untreated) and RT-qPCR (ii–v), which was normalised to tubulin mRNA. **(B)** EndoC-βH3 cells were cotransfected with *Renilla*-HBB(PTC^-) and/or *Renilla*-HBB(PTC^+) and the *Firefly* plasmid and exposed to PBS as untreated (Unt), cytokine combination (Cyt; 3 ng/mL IL-1β + 10 ng/mL IFN-γ+ 10 ng/mL TNF-α) alone, and/or simultaneously with 16 μM of 4μ8C, an endoribonuclease inhibitor of IRE1α, and/or 8 μM of GSK2656157 (GSK157), PERK inhibitor for 18 (h) (**B**(i)) Luciferase activity was measured in the lysate of EndoC-βH3 cells transfected with *Renilla*-HBB(PTC^-) and or *Renilla*-HBB(PTC^+) and the *Firefly* plasmid exposed to PBS as untreated (Unt) or given conditions, and represented as NMD activity calculated by dividing luciferase activity of HBB (PTC^-)/HBB(PTC^+) as explained in the Methods. (ii) mRNA level of *Renilla-HBB*-fused gene and *Firefly* gene in the EndoC-βH3 cells was quantified by RT-qPCR using specific primers extending the junction of exons 1 and 2 of the *HBB* gene and the *Renilla* gene, or only *Firefly* gene, and normalised to tubulin. The data are means ± SEM of $N = 6$. The symbol "*" indicates the Bonferroni-corrected paired *t*-test values of treated versus untreated (Unt) **(A, B)** or, otherwise, cytokines (Cyt) that are designated by a line on top of the bars **(B)**: *≤ 0.05; **≤ 0.01; ***≤ 0.001; ****≤ 0.0001. FDR, false discovery rate; RLuc, *Renilla* luciferase; Ex, exon.

Cytokine-induced suppression of NMD activity is associated with UPF3B downregulation and is attenuated by UPF3 overexpression in β cells

Since we observed in our previous study that cytokine-induced ER stress downregulated UPF3B expression in human and rodent β cells, as recovering nitroxidative-driven ER stress using the inducible nitric oxide synthase (iNOS) inhibitor *N*-methyl-l-arginine (NMA) (11), since transcripts encoding UPR components are NMD targets and have been shown to be stabilised by UPF3A/B depletion (19), and since UPF3B is a NMD activator in mammalian cells (23), which led to the proposal of UPF3-dependent and UPF3-independent branches of the NMD pathway (4, 10, 24). We reasoned that UPF3 regulated NMD activity in β cells. We therefore first measured the UPF3A/B expression level and then investigated the functional impact of overexpressing UPF3A/B on cytokine-mediated suppression of NMD activity in β cells. RT-qPCR examination showed that cytokines significantly downregulated UPF3B mRNA levels after 18 h in both EndoC-βH3 (Figures 3Ai, ii) and INS1(832/13) [Supplementary Figures S4A (i, ii)] as previously reported (11). Immunoblot analysis verified overexpression of UPF3A, UPF3B, and the UPF3B dominant negative UPF3BΔ42 in both EndoC-βH3 [Supplementary Figure S4B (i)] and INS1(832/13) [Supplementary Figure S4C (i)]. Cytokines reduced NMD activity, and overexpression of UPF3B significantly attenuated this reduction in EndoC-βH3 [Figure 3B; Supplementary Figure S4B (ii)] and to a lesser extent in INS1(832/13) [Supplementary Figures S4C (ii, iii)]. Neither UPF3A nor UPF3BΔ42 overexpression counteracted cytokine-attenuated NMD activity.

This result suggests that cytokines reduce NMD activity in β cells through downregulation of UPF3B expression.

UPF3 overexpression deteriorates cell viability and reduces insulin content but not secretion in EndoC-βH3 cells

The above findings provide evidence that the UPF3-dependent branch of NMD is involved in cytokine-mediated suppression of NMD activity. Therefore, we next investigated the impact of UPF3A/B overexpression on cytokine-induced cell death and insulin secretion. While UPF3A or UPF3B overexpression increased basal cell death, it also exacerbated the cytokine-induced apoptosis in EndoC-βH3 cells (Figures 4Ai, ii). In INS1 (832/13) cells, neither UPF3A nor UPF3B overexpression changed cell viability in the absence of Cyt exposure, but UPF3B overexpression significantly aggravated cytokine-induced cell death as measured by Alamarblue and caspase-3 activity assays [Supplementary Figures S5A (i, ii)]. Therefore, we next explored the impact of UPF3A or UPF3B deficiency on β-cell viability. Lentiviral shRNA-mediated knockdown of UPF3A and/or UPF3B [Supplementary Figures S5B (i, ii)] significantly reduced basal INS1(832/13) cell viability (Supplementary Figure S5C (i–iv). Taken together, these data indicate that genetic manipulations of UPF3A/B could possibly be detrimental to the β-cell viability.

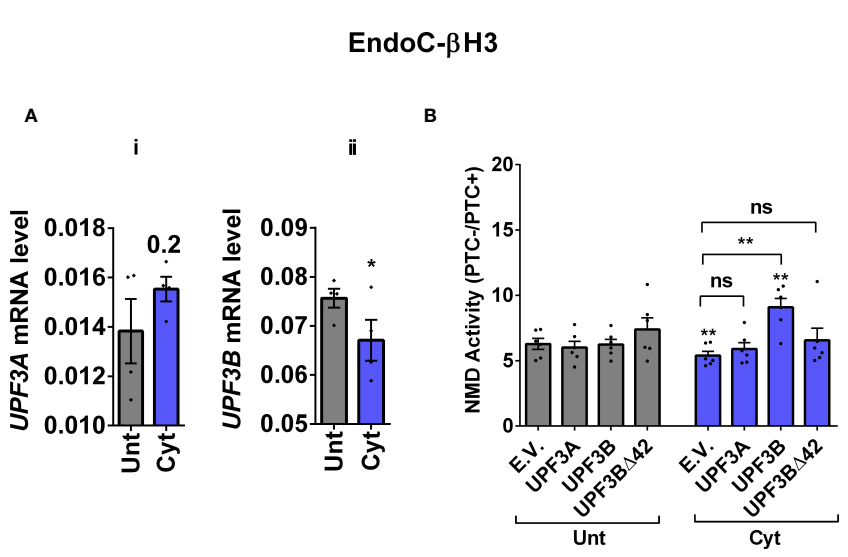

FIGURE 3
Cytokine-induced suppression of NMD activity is associated with UPF3B downregulation and attenuated by UPF3 overexpression in β cells. EndoC-βH3 cells were cotransfected with empty vector (E.V.), UPF3A, UPF3B, and/or UPF3BΔ42 (dominant negative of UPF3B) plasmids, and then with *Renilla*-HBB(PTC$^-$) and or *Renilla*-HBB(PTC$^+$), along with the *Firefly* plasmid and exposed to cytokine combination (Cyt; 3 ng/mL IL-1β + 10 ng/mL IFN-γ + 10 ng/mL TNF-α) for 18 (h) **(A**(i, ii)**)** mRNA level of *Upf3A* and *Upf3B* genes in EndoC-βH3 cells was quantified by RT-qPCR and normalised to tubulin mRNAs. **(B)** Luciferase activity was measured in the lysate of the transfected cells and represented as NMD activity calculated by dividing luciferase activity of HBB(PTC$^-$)/HBB(PTC$^+$) as explained in the Methods. The overexpression of UPF3A and UPF3B proteins was examined by Western blot analysis [Supplementary Figure 4B (i)]. The data are means ± SEM of $N = 6$. The symbol "*" indicates the Bonferroni-corrected paired *t*-test values of treated versus untreated E.V. (Unt) **(A, B)** or, otherwise, cytokine (Cyt)-treated E.V. that is designated by a line on top of the bars **(B)**: *≤ 0.05; **≤ 0.01. ns, nonsignificant.

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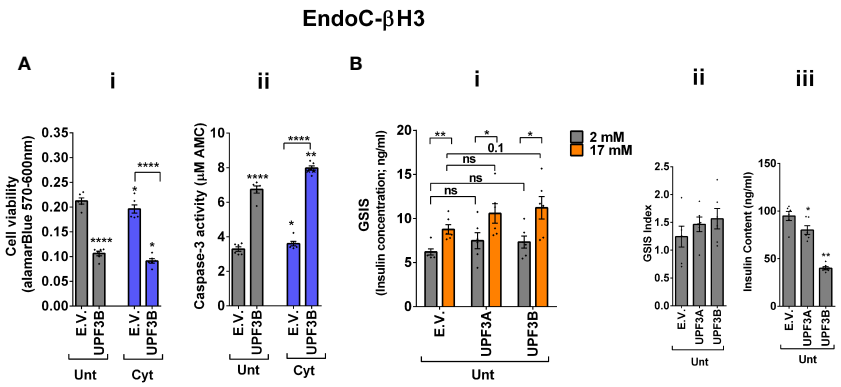

FIGURE 4
UPF3 overexpression deteriorates cell viability and reduces insulin content but not secretion in EndoC-βH3 cells. EndoC-βH3 cells were cotransfected with empty vector (E.V.), UPF3A, and/or UPF3B plasmids and exposed to cytokine combination (Cyt; 3 ng/mL IL-1β + 10 ng/mL IFN-γ + 10 ng/mL TNF-α) for 3 days. **(A)** Cell viability was measured by Alamarblue (i) and caspase-3 activity (ii) assays (N = 6). **(B)** Glucose-stimulated insulin secretion (GSIS) (i) and insulin contents (iii) were investigated in the transfected EndoC-βH3 cells. Insulin concentration (ng/mL) was measured by insulin ultrasensitive assay (N = 6). GSIS index (ii) was calculated by dividing the insulin concentration measured in the treatments at 17 mM by 2 mM of glucose. The data are means ± SEM of N = 6. The symbol "*" indicates the Bonferroni-corrected paired t-test values of treated versus untreated E.V. (Unt) or, otherwise, cytokine (Cyt)-treated E.V. that is designated by a line on top of the bars **(A)** or the Bonferroni-corrected paired t-test values of the corresponding low versus high glucose, that is otherwise designated by lines on top of the bars **(B**(i)**)**: *≤ 0.05; **≤ 0.01; ****≤ 0.0001. ns, nonsignificant.

In contrast to Cyt, which downregulates UPF3B in INS1 (11), EndoC-βH3 (Figure 3Aii), and INS1(832/13) cells [Supplementary Figure S4A (ii)], GLT does not downregulate UPF3B (11).We therefore explored the effect of UPF3 deficiency on glucolipotoxicity-induced cell death in β cells. Measurements of caspase-3 activity demonstrated that both UPF3A and UPF3B knockdown rendered a slight but significant protection against 24 h glucolipotoxicity in INS1(832/13) cells in comparison with untreated cells [Supplementary Figure S5D (i, ii)]. Furthermore, treatment with the NMD activator Tranilast dose-dependently sensitised to glucolipotoxicity-, but not cytokine-induced, EndoC-βH3 cell death, measured by caspase-3 activity assay [Supplementary Figure S4E (i, ii)]. Neither UPF3A nor UPF3B overexpression affected GSIS in EndoC-βH3 (Figure 4Bi, ii) or INS1(832/13) [Supplementary Figure S5F (I, ii)] cells. Nonetheless, UPF3B overexpression profoundly lowered insulin content in EndoC-βH3 cells (Figure 4Biii). In contrast, knockdown of UPF3A or UPF3B significantly decreased the stimulatory index, as well as provoking a substantial increase in insulin content in control INS1(832/13) cells [Supplementary Figures S5G (ii, iii), H (ii and iii)], without altering ins1 or ins2 mRNA expression [Supplementary Figures S5G (iv, v), H (iv, v)].

Taken together, these findings reveal that UPF3 overexpression induces basal cell death and exacerbates cytokine-mediated toxicity in β cells.

UPF2 knockdown potentiates cytokine suppression of NMD activity and slightly alleviates cytokine toxicity for cell viability and insulin content in EndoC-βH3 cells

Next, we investigated the effect of UPF2 deficiency on the viability and insulin secretion of β cells because (i) UPF3A and UPF3B are involved in regulating UPF2, a key core NMD activator in mammalian cells (25, 26) by sequestering away from and bridging the exon-junction complex (EJC) with UPF1 and UPF2, respectively, leading to the NMD activation (23), and genome-wide association (GWAS) data reveal that the *UPF2* variant rs145580445 is significantly associated with type 2 diabetes risk (7). We, therefore, knocked down the *UPF2* gene in EndoC-βH3 cells using RNA interference and chose the three cell lines in which UPF2 was most efficiently knocked down (KD) [Supplementary Figures S6A (i, ii)]. Examination of NMD activity using the luciferase-based NMD reporter revealed that UPF2 KD profoundly reduced NMD activity in untreated and cytokine-treated EndoC-βH3 cells [Supplementary Figures S6B (i, ii)]. Compared with NS control, UPF2 KD slightly but significantly prevented cytokine-induced cell death (Figures 5Ai, ii). UPF2 KD had no effect on the GSIS but significantly increased insulin content (Figures 5Bi–iii).

These data indicate that UPF2 plays a crucial role in cytokine-induced β-cell apoptosis. In addition, the increase in insulin content in UPF2-deficient EndoCβH3 cells implies that insulin transcripts could possibly be targets of the UPF2-dependent NMD pathway branch.

UPF2 knockdown differentially affects cytokine- and glucolipotoxicity-mediated deregulation of EndoC-βH3 transcripts

Consistent with our observations above (Figure 5), we previously reported (11) that the deficiency of SMG6, an endoribonuclease and a key effector of NMD, rendered protection against cytokine-induced cell death and was associated with increased insulin content. Therefore, we aimed to identify

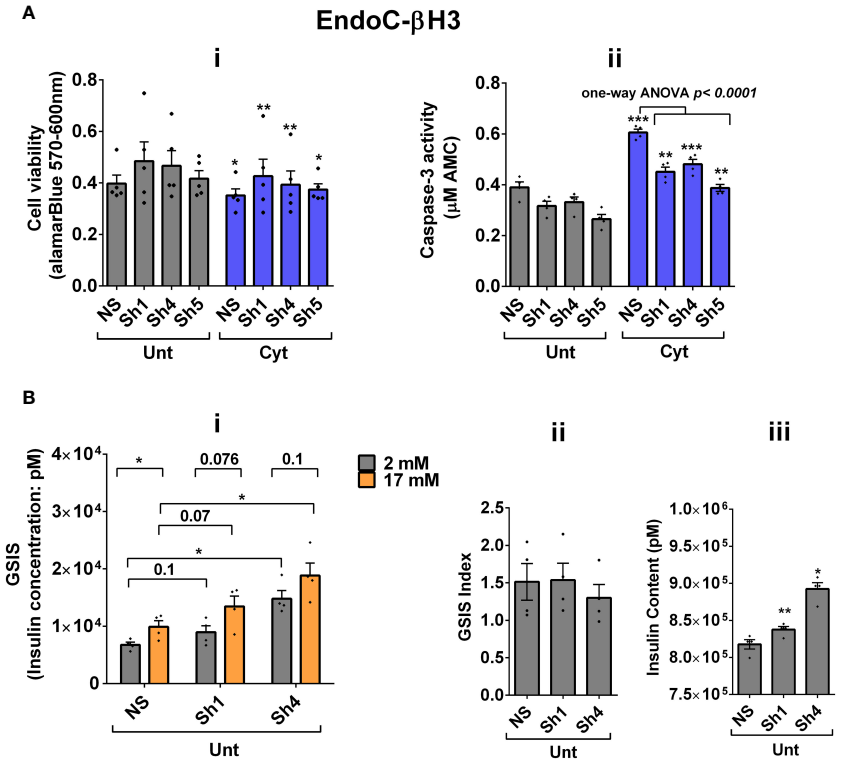

FIGURE 5
UPF2 knockdown potentiates cytokine suppression of NMD activity and slightly alleviates cytokine toxicity for cell viability and insulin content in EndoC-βH3 cells. EndoC-βH3 cell lines with the most efficient stable knockdown (KD) of UPF2 (three shRNAs; Sh1, Sh4, and Sh5) and nonsilencing shRNA control (NS) were cotransfected with *Renilla*-HBB(PTC$^-$) and or *Renilla*-HBB(PTC$^+$) and the *Firefly* plasmids and exposed to PBS as untreated (Unt) and or cytokine combination (Cyt; 3 ng/mL IL-1β + 10 ng/mL IFN-γ+10 ng/mL TNF-α). **(A)** Cell viability was measured by Alamarblue (i) and caspase-3 activity (ii) assays (N = 6). **(B)** Glucose-stimulated insulin secretion (GSIS) (i) and insulin contents (iii) were investigated in the UPF2 KD EndoC-βH3 cells. Insulin concentration (pM) was measured by human insulin ELISA (N = 6). GSIS index (ii) was calculated by dividing insulin concentration measured in the treatments of 17 mM by 2 mM glucose. The data are means ± SEM. The symbol "*" indicates the Bonferroni-corrected paired t-test values of treated versus untreated (Unt) NS control (**A**, **B**), otherwise designated by a line on top of the bars, or the Bonferroni-corrected paired t-test values of corresponding low versus high glucose, that is, otherwise designated by lines on the top of the bars (**B**(i)). *≤ 0.05; **≤ 0.01; ***≤ 0.001.

potential NMD target transcripts by using RNA-sequencing to assess the transcriptome of cytokine- or PBS-treated EndoC-βH3 cells stably transfected with a nonsilencing shRNA (NS) or the specific shRNA (shRNA-1 named U1) against *UPF2*. Due to the differential effects of Cyt and GLT on UPF3 expression cf. above, we also performed RNA-sequencing after UPF2 KD versus NS control EndoC-βH3 exposed to GLT compared with PBS-treated.

The RNA-seq datasets from either UPF2 KD or NS control EndoC-βH3 cell lines exposed to cytokines and or GLT were dimensionally reduced by principal component analysis (PCA) into two main principal components, PC1 and PC2 ($p < 0.05$). The PCA of the NS control EndoC-βH3 cells demonstrated a high similarity between the biological replicates, a small within-group variance, and a distinct clustering of the untreated cytokine and GLT groups [Supplementary Figures S7A (i, ii)]. Pearson's correlation ($p < 0.05$) between samples justified the clustering of biological replicates of cytokines, GLT, and untreated conditions [Supplementary Figures S7B (i, ii)]. PCA revealed that the UPF2 knockdown increased the majority of variance in transcript isoforms of the cell, which led to patterns that visually dispersed biological replicates from the cytokine-exposed isolates, while decreasing the variances from the GLT and untreated biological replicates, which clustered them together.

A Venn diagram of the RNA-seq datasets demonstrated an approximate total of 14,000 commonly expressed transcripts (FDR < 0.05) and those differentially expressed transcripts regulated by PBS (i.e., untreated control), cytokines, or GLT [Supplementary Figures S7C (i, ii), D (i–iv)]. Among them, cytokines regulated 54% (up) and 46% (down) (Figure 6Ai), whereas GLT impacted 48% (up) and 52% (down) of significantly expressed transcripts in NS control EndoC-βH3 cells (Figure 6Aiv). UPF2 KD changed the cytokine-mediated regulation of significantly expressed transcripts by 59% (up) and 41% (down) (Figure 6Aii), whereas it did not change the regulation of significantly expressed transcripts by GLT (up: 47%, down: 53%) in EndoC-βH3 cells (Figure 6Av). This indicates that UPF2 KD possibly alters the mRNA levels of identified transcript species.

To identify UPF2 KD-regulated transcripts possibly providing protection against cytokine-induced cytotoxicity, we interrogated cytokine- and/or GLT-regulated transcripts that were differentially expressed ($p < 0.05$) in UPF2 KD versus NS control EndoC-βH3 cells using gene enrichment analysis (GEA). Gene Ontology (GO)

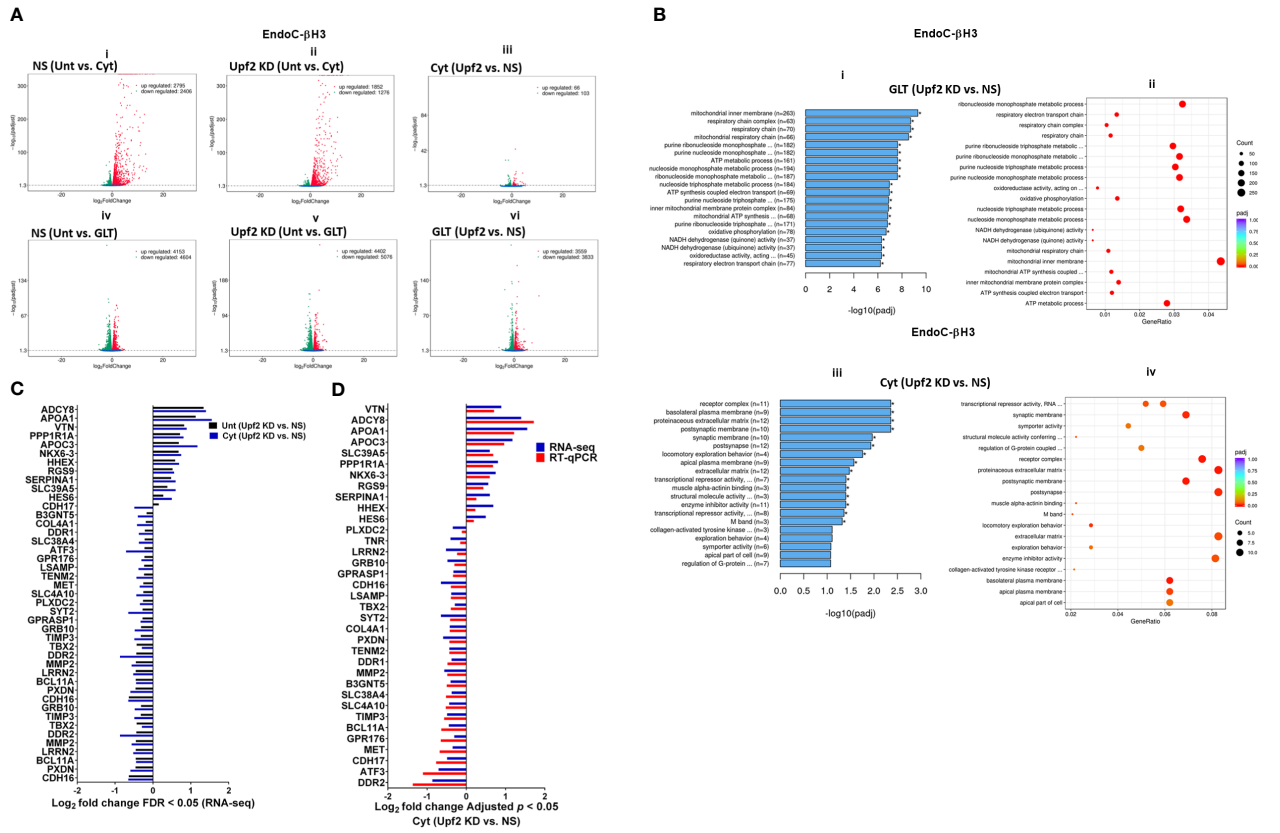

FIGURE 6
UPF2 knockdown differentially affects cytokine- and glucolipotoxicity-mediated deregulation of EndoC-βH3 transcripts. EndoC-βH3 cell lines knocked down for UPF2 (shRNA-1 named U1) or nonsilencing shRNA control (NS) were exposed to PBS as untreated (Unt), cytokine combination (Cyt; 3 ng/mL IL-1β + 10 ng/mL IFN-γ + 10 ng/mL TNF-α), and/or glucolipotoxicity (GLT; 0.5 mM palmitate +25 mM glucose). Total RNA was extracted from the treated cells, and cDNA library was made and sequenced using the Hiseq platform as explained in the Methods. In total, 33 RNA-seq datasets from NS/CTL (N = 6), NS/Cyt (N = 6), NS/GLT (N = 4), U1/CTL (N = 6), U1/Cyt (N = 6) and U1/GLT (N = 5) were analysed through the pipeline described in the Supplementary Methods. **(A)** Volcano plot of the number of transcripts (FDR < 0.05) regulated by Cyt or GLT versus untreated in the NS or UPF2 KD (U1) cell lines. **(B)** Top enriched pathways (i, iii) and the number of transcripts (ii, iv) regulated by Cyt and/or GLT in the UPF2 KD (FDR < 0.0.5). Enrichment is shown as log10 (adjusted p-value < 0.05). **(C)** Top GEA-identified transcripts regulated by Cyt compared to untreated in UPF2 KD versus NS control EndoC-βH3 cells. The expression level is shown as log2 (adjusted p-value < 0.05). **(D)** RT-qPCR verification of the identified transcripts regulated by Cyt in UPF2 KD versus NS control EndoC-βH3 cells. The expression level is shown as log2 (adjusted p-value < 0.05).

and Kyoto Encyclopaedia of Genes and Genomes (KEGG) gene enrichment analyses demonstrated that GLT significantly ($p < 0.05$) regulated transcripts in the cellular functions of RNA splicing, mitochondrial inner membrane, and purine nucleoside metabolism [Supplementary Figure S7E (i, ii)], although these were not affected by UPF2 KD (Figure 6Bi). In contrast, GEA revealed that in both untreated and cytokine-treated EndoC-βH3 cells, UPF2 KD significantly regulated transcripts encoding proteins involved in synaptic transmission, extracellular matrix, basolateral plasma membrane, receptor complex, synaptic membrane, transcriptional repressor activity, and enzyme inhibitor activity [Figures 6Biii, C; Supplementary Figures S7E (iii, iv)].

RT-qPCR confirmed the logarithmic fold change of UPF2 KD-regulated transcripts in the cytokine-treated EndoC-βH3 cells versus cytokine-treated NS control cells (Figure 6D). The role of many of these transcripts in cell viability or insulin secretion was previously identified in pancreatic β cells. Among them, α-1-antitrypsin (1-AT) has been proposed as an antagonist against cytokine-induced pancreatic β-cell death (27, 28). Our expression profiling verified that cytokines upregulated α-1-antitrypsin, and this effect was further potentiated by UPF2 KD. To explore the potential importance of these changes, we knocked down α-1-antitrypsin using specific siRNAs in INS1(832/13) and EndoC-βH3 cells, as confirmed by quantitative WB [Supplementary Figure S8 (Fig.7B-i, ii and iii)]. The effect of siRNA-mediated α-1-antitrypsin knockdown was inconclusive in EndoC-βH3 cells (Figure 7Ai, ii) [Supplementary Figures S7B (i, ii)]. Both α-1-antitrypsin siRNAs aggravated cytokine-induced cell death in comparison with NS control in INS1(832/13) cells (Figures 7Aiii, iv). Compared with the NS control, α-1-antitrypsin knockdown reduced the GSIS index but had no effect on insulin content in INS1(832/13) cells (Figures 7Bi, ii, iii), possibly due to the reduced cell number.

Cytokines reportedly upregulate > 30 splicing factors, affecting alternative splicing of 35% of genes in the human islet transcriptome (6). We examined RNA-seq datasets for alternative splicing (AS) isoforms [Supplementary Figure S9 (i)] driven by

cytokines or GLT versus untreated in the NS control and UPF2 KD EndoC-βH3 cells. Among 2,123 and 2,106 cytokine-driven AS isoforms, skipped exon (SE) isoforms constituted 70.89% (p = 0.1, n = 6) and 72.5% (p = 0.1, n = 6) in NS control and UPF2 KD cells, respectively [Supplementary Figures S9 (ii, iii)]. In contrast, 220 and 133 GLT-driven AS isoforms were identified in NS control and UPF2 KD, respectively [Supplementary Figures S9 (iv, v)]. This differential regulation could possibly provide a reliable measure for cytokines and the role of GLT in inducing AS isoforms in β cells.

Taken together, the above transcriptome analysis of EndoC-βH3 cells indicates that cytokines increase 1–AT expression, and this is synergised by NMD attenuation.

Discussion

In this study, we demonstrate that cytokines decrease NMD in INS1(832/13), EndoC-βH3 cells, and dispersed human islets. We also showed that the cytokine-mediated decrease of NMD activity was driven by ER stress and downregulation of UPF3B. Loss-/or gain-of-function of NMD activity could be elicited by UPF3B overexpression or UPF2 knockdown, which led to increases in, or slight decreases in, cytokine-induced apoptosis associated with decreased and increased insulin contents, respectively, without affecting GSIS index in EndoC-βH3 cells. Transcriptome profiling indicated a potentiating effect of UPF2 knockdown on Cyt, but not GLT-mediated, NMD activity. Interestingly, this approach identified transcript targets encoding proteins belonging to the extracellular matrix such as α-1-antitrypsin. Importantly, the knockdown of this gene enhanced cytokine-induced cytotoxicity in β cells.

To the best of our knowledge, the present study represents the first demonstration of a functional effect of cytokines on NMD activity.

UPR activation is known to inhibit NMD via PERK activation and eIF2α phosphorylation to restore IRE1α accumulation and hence a robust UPR activation (19, 20, 29); in addition to the role of PERK activation, our findings suggest that IRE1α riboendonuclease activity (p = 0.1) was involved in cytokine-mediated NMD inhibition in EndoC-βH3 cells.

UPF3A and UPF3B act as a potent NMD inhibitors and activators, respectively, in HeLa cells and in mice (23), consistent with our observations following UPF3B overexpression in β cells. However, the finding that forced UPF3A overexpression slightly increased NMD activity in β cells seems inconsistent with previous findings. Recent studies (30, 31) support our apparently discrepant finding regarding the effects of UPF3A overexpression by showing the redundancy of UPF3A and UPF3B as modular activators of

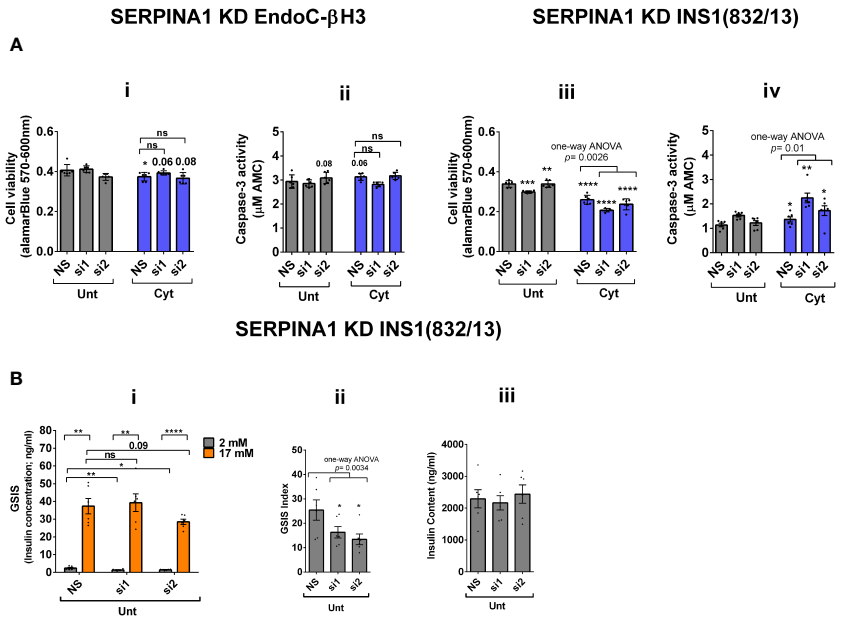

FIGURE 7
SERPINA1 knockdown deteriorates cytokine cytotoxicity for viability and glucose-stimulated insulin secretion index in INS1(832/13) cells. EndoC-βH3 and INS1(832/13) cells were transfected with siRNAs against SERPINA1 (two species-specific siRNAs for each cell type; si1 and si2) and a nonsilencing siRNA control (NS), incubated for 24 h and exposed to PBS as untreated (Unt) and cytokine combination (Cyt for EndoC-βH3; 3 ng/mL IL-1β + 10 ng/mL IFN-γ+ 10 ng/mL TNF-α) (Cyt for INS1(832/13); 150 pg/mL IL-1β + 0.1 ng/mL IFN-γ+ 0.1 ng/mL TNF-α) for 72 and 24 h, respectively (see Supplementary Methods). The knockdown efficiency was checked using quantitative WB [Supplementary Figures S8 (i–iii)]. **(A)** Cell viability was measured by Alamarblue (i, iii) and caspase-3 activity (ii, iv) assays (N = 6). **(B)** Glucose-stimulated insulin secretion (GSIS) (i) and insulin contents (iii) were investigated in the transfected EndoC-βH3 cells. Insulin concentration (ng/mL) was measured by insulin ultrasensitive assay (N = 6). GSIS index (ii) was calculated by dividing insulin concentration measured in the treatments of 17 mM by 2 mM glucose. The data are means ± SEM of N = 6. The symbol "*" indicates the Bonferroni-corrected paired t-test values of treatments versus untreated (Unt) NS control or cytokine (Cyt)-treated NS that is, otherwise, designated by a line on top of the bars **(A)**, or corresponding low versus high glucose that is, otherwise, designated by lines on the top of the bars (**B**(i)): *≤ 0.05; **≤ 0.01; ***≤ 0.001; ****≤ 0.0001. ns, nonsignificant.

NMD (24). With these two earlier studies in mind, we cannot rule out the interference of endogenous UPF3A in the actions of UPF3B on NMD in β cells.

We investigated the consequences of NMD activity on pancreatic β-cell function and viability. The increase in NMD activity by UPF3B overexpression induced basal and cytokine-induced cell death in EndoC-βH3 cells, highlighting the role of increased UPF3B levels in β cells. This appears to be relevant for β-cell viability in both normal and inflammatory stress conditions. Similarly, UPF3B knockdown also caused basal cell death in INS1 cells. Hence, basal UPF3A/B levels seem to play crucial roles in the cell viability of β cells, and perturbation of such a controlled level implicates cell death. On the other hand, the slight protection against cytokine-induced cell death conferred by UPF2 knockdown in EndoC-βH3 cells (Figures 5Ai, ii) and by SMG6 knockdown in INS1 cells (11) implies a possibly protective mechanism against the cytotoxicity of cytokines in β cells, irrespective to the outcome cytokine-induced cell death. Moreover, our findings provide evidence that the reduced insulin content observed after UPF3B overexpression is related to overactivated NMD.

Cytokine-induced perturbation of NMD (potentiated by UPF2 silencing) might change the balance of anti-/proapoptotic transcripts. This, in turn, may contribute to cytotoxic damage. Consistent with this view, GEA revealed that cytokines deregulate transcripts encoding proteins that localise to and/or function in the extracellular matrix. Thus, α-1-antitrypsin knockdown increased the detachment of MIN6 cells and exacerbated thapsigargin-induced cell death as measured by propidium-iodide staining (28) and, in this study, increased cytokine-induced cell death in INS1 (832/13) cells associated with decreased GSIS index.

We speculate that the perturbation of NMD by cytokines leads to increased exon skipping and that this may be part of a feedback loop promoting β-cell plasticity and resilience against cytotoxic cytokines. Future studies will be needed to test this possibility. We note also that depletion of alternative splicing factors (reviewed in (3)) inhibits insulin secretion and induces basal apoptosis after treatment with cytokines in rodent and human β cells (3, 7, 32, 33). Moreover, antisense-mediated exon skipping of 48–50 exons of the dystrophin gene restores the open reading frame and allows the generation of partially to largely functional proteins (34).

In conclusion, we reveal that cytokines suppress NMD activity via ER stress signalling, possibly as a protective response against cytokine-induced NMD component expression. Our findings highlight the central importance of RNA turnover in β-cell responses to inflammatory stress.

Limitations and future perspectives

We used a luciferase-based NMD reporter based on two separate PTC^- and PTC^+ constructs whose labelled luciferase is separately measured. Thus, a yet-to-develop NMD activity reporter by which transcript RNA, protein, or their corresponding labelled luciferase activity of both PTC^- and PTC^+ transcripts could be examined in one cell rather than (potentially) two separate cells will remove the limitation of the current reporter based on the transfection of the constructs into two separate cells. Moreover, the constant overexpression of UPF3A and UPF3B may result in cell death as β cells cannot cope with the overwhelming levels of these proteins above the basal level. This could explain why UPF3B overexpression reduces the basal cell viability. Further studies should (1) conduct *in vivo* experiments to validate the findings observed *in vitro* and determine if the regulation of NMD in pancreatic β cells is consistent across different cell types and conditions; (2) investigate the mechanisms underlying the regulation of NMD in pancreatic β cells, including the role of specific NMD components and the impact of different stressors on NMD activity; and (3) explore the potential therapeutic implications of targeting NMD in the treatment of inflammatory stress in β cells, including the development of novel drugs or therapies that modulate NMD activity.

Translatability of the findings

The findings report NMD involvement in the development of islet autoimmunity and the destruction of pancreatic β cells in type 1 diabetes, as well as islet inflammation in type 2 diabetes. The identification of novel targets arising from cytokine-driven NMD attenuation could possibly suggest new biomarkers to monitor disease progression and may also guide the development of protein-based vaccines or antisense mRNA therapeutics in individuals who are at risk of diabetes development and or other inflammatory and autoimmune disorders.

Data availability statement

The RNA-seq data from the human insulin-producing cell line EndoC-βH3 that support the findings of Figure 6 of this study are deposited in the Sequence Read Archive (SRA) data repository (Accession numbers for 33 RNA-seq datasets: SRR22938756-SRR22938788) under the BioProject accession number (PRJNA916946) that are appreciated for further citations.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. Ethical approval was not required for the studies on animals in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. Islet isolation was approved by the Human Research Ethics Board at the University of Alberta (Pro00013094). All donors' families gave informed consent for the use of pancreatic tissue in research.

Author contributions

SG: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. PM: Funding acquisition, Resources, Writing – original draft, Writing – review & editing. LP: Resources, Writing – original draft, Writing – review & editing. JN: Methodology, Writing – original draft, Writing – review & editing. BP: Investigation, Writing – original draft, Writing – review & editing. TM-P: Writing – original draft, Writing – review & editing. GR: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

Author GR is a consultant for, and has received grant funding from, Sun Pharmaceuticals Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1359147/full#supplementary-material

References

1. Rutter GA, Georgiadou E, Martinez-Sanchez A, Pullen TJ. Metabolic and functional specialisations of the pancreatic beta cell: gene disallowance, mitochondrial metabolism and intercellular connectivity. *Diabetologia*. (2020) 63:1990–8. doi: 10.1007/s00125-020-05205-5

2. Eizirik DL, Pasquali L, Cnop M. Pancreatic beta-cells in type 1 and type 2 diabetes mellitus: different pathways to failure. *Nat Rev Endocrinol*. (2020) 16:349–62. doi: 10.1038/s41574-020-0355-7

3. Alvelos MI, Juan-Mateu J, Colli ML, Turatsinze JV, Eizirik DL. When one becomes many-Alternative splicing in beta-cell function and failure. *Diabetes Obes Metab*. (2018) 20 Suppl 2:77–87. doi: 10.1111/dom.13388

4. Huang L, Wilkinson MF. Regulation of nonsense-mediated mRNA decay. *Wiley Interdiscip Rev RNA*. (2012) 3:807–28. doi: 10.1002/wrna.1137

5. Le K, Mitsouras K, Roy M, Wang Q, Xu Q, Nelson SF, et al. Detecting tissue-specific regulation of alternative splicing as a qualitative change in microarray data. *Nucleic Acids Res*. (2004) 32:e180. doi: 10.1093/nar/gnh173

6. Eizirik DL, Sammeth M, Bouckenooghe T, Bottu G, Sisino G, Igoillo-Esteve M, et al. The human pancreatic islet transcriptome: expression of candidate genes for type 1 diabetes and the impact of pro-inflammatory cytokines. *PloS Genet*. (2012) 8: e1002552. doi: 10.1371/journal.pgen.1002552

7. Ghiasi SM, Rutter GA. Consequences for pancreatic beta-cell identity and function of unregulated transcript processing. *Front Endocrinol (Lausanne)*. (2021) 12:625235. doi: 10.3389/fendo.2021.625235

8. Hug N, Longman D, Caceres JF. Mechanism and regulation of the nonsense-mediated decay pathway. *Nucleic Acids Res*. (2016) 44:1483–95. doi: 10.1093/nar/gkw010

9. Pan Q, Saltzman AL, Kim YK, Misquitta C, Shai O, Maquat LE, et al. Quantitative microarray profiling provides evidence against widespread coupling of alternative splicing with nonsense-mediated mRNA decay to control gene expression. *Genes Dev*. (2006) 20:153–8. doi: 10.1101/gad.1382806

10. Chang YF, Imam JS, Wilkinson MF. The nonsense-mediated decay RNA surveillance pathway. *Annu Rev Biochem*. (2007) 76:51–74. doi: 10.1146/annurev.biochem.76.050106.093909

11. Ghiasi SM, Krogh N, Tyrberg B, Mandrup-Poulsen T. The no-go and nonsense-mediated RNA decay pathways are regulated by inflammatory cytokines in insulin-producing cells and human islets and determine beta-cell insulin biosynthesis and survival. *Diabetes*. (2018) 67:2019–37. doi: 10.2337/db18-0073

12. Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB. Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology*. (1992) 130:167–78. doi: 10.1210/endo.130.1.1370150

13. Ravassard P, Hazhouz Y, Pechberty S, Bricout-Neveu E, Armanet M, Czernichow P, et al. A genetically engineered human pancreatic beta cell line exhibiting glucose-inducible insulin secretion. *J Clin Invest*. (2011) 121:3589–97. doi: 10.1172/JCI58447

14. Boelz S, Neu-Yilik G, Gehring NH, Hentze MW, Kulozik AE. A chemiluminescence-based reporter system to monitor nonsense-mediated mRNA decay. *Biochem Biophys Res Commun*. (2006) 349:186–91. doi: 10.1016/j.bbrc.2006.08.017

15. Neu-Yilik G, Raimondeau E, Eliseev B, Yeramala L, Amthor B, Deniaud A, et al. Dual function of UPF3B in early and late translation termination. *EMBO J*. (2017) 36:2968–86. doi: 10.15252/embj.201797079

16. Cunningham F, Allen JE, Allen J, Alvarez-Jarreta J, Amode MR, Armean IM, et al. Ensembl 2022. *Nucleic Acids Res*. (2022) 50:D988–D95. doi: 10.1093/nar/gkab1049

17. Ghiasi SM, Hansen JB, Christensen DP, Tyrberg B, Mandrup-Poulsen T. The connexin 43 regulator rotigaptide reduces cytokine-induced cell death in human islets. *Int J Mol Sci*. (2020) 21(12):4311. doi: 10.3390/ijms21124311

18. Vishnu N, Hamilton A, Bagge A, Wernersson A, Cowan E, Barnard H, et al. Mitochondrial clearance of calcium facilitated by MICU2 controls insulin secretion. *Mol Metab*. (2021) 51:101239. doi: 10.1016/j.molmet.2021.101239

19. Karam R, Lou CH, Kroeger H, Huang L, Lin JH, Wilkinson MF. The unfolded protein response is shaped by the NMD pathway. *EMBO Rep*. (2015) 16:599–609. doi: 10.15252/embr.201439696

20. Goetz AE, Wilkinson M. Stress and the nonsense-mediated RNA decay pathway. *Cell Mol Life Sci*. (2017) 74:3509–31. doi: 10.1007/s00018-017-2537-6

21. Csutora P, Su Z, Kim HY, Bugrim A, Cunningham KW, Nuccitelli R, et al. Calcium influx factor is synthesized by yeast and mammalian cells depleted of organellar calcium stores. *Proc Natl Acad Sci U.S.A*. (1999) 96:121–6. doi: 10.1073/pnas.96.1.121

22. Sehgal P, Szalai P, Olesen C, Praetorius HA, Nissen P, Christensen SB, et al. Inhibition of the sarco/endoplasmic reticulum (ER) Ca(2+)-ATPase by thapsigargin analogs induces cell death via ER Ca(2+) depletion and the unfolded protein response. *J Biol Chem*. (2017) 292:19656–73. doi: 10.1074/jbc.M117.796920

23. Shum EY, Jones SH, Shao A, Dumdie J, Krause MD, Chan WK, et al. The antagonistic gene paralogs upf3a and upf3b govern nonsense-mediated RNA decay. *Cell*. (2016) 165:382–95. doi: 10.1016/j.cell.2016.02.046

24. Yi Z, Arvola RM, Myers S, Dilsavor CN, Abu Alhasan R, Carter BN, et al. Mammalian UPF3A and UPF3B can activate nonsense-mediated mRNA decay independently of their exon junction complex binding. *EMBO J*. (2022) 41(10):e109202. doi: 10.15252/embj.2021109202

25. Serdar LD, Whiteside DL, Baker KE. ATP hydrolysis by UPF1 is required for efficient translation termination at premature stop codons. *Nat Commun*. (2016) 7:14021. doi: 10.1038/ncomms14021

26. Neu-Yilik G, Gehring NH, Hentze MW, Kulozik AE. Nonsense-mediated mRNA decay: from vacuum cleaner to Swiss army knife. *Genome Biol*. (2004) 5:218. doi: 10.1186/gb-2004-5-4-218

27. Lewis EC, Mizrahi M, Toledano M, Defelice N, Wright JL, Churg A, et al. alpha1-Antitrypsin monotherapy induces immune tolerance during islet allograft transplantation in mice. *Proc Natl Acad Sci U.S.A*. (2008) 105:16236–41. doi: 10.1073/pnas.0807627105

28. McKimpson WM, Chen Y, Irving JA, Zheng M, Weinberger J, Tan WLW, et al. Conversion of the death inhibitor ARC to a killer activates pancreatic beta cell death in diabetes. *Dev Cell*. (2021) 56:747–60 e6. doi: 10.1016/j.devcel.2021.02.011

29. Karousis ED, Nasif S, Muhlemann O. Nonsense-mediated mRNA decay: novel mechanistic insights and biological impact. *Wiley Interdiscip Rev RNA*. (2016) 7:661–82. doi: 10.1002/wrna.1357

30. Wallmeroth D, Lackmann JW, Kueckelmann S, Altmüller J, Dieterich C, Boehm V, et al. Human UPF3A and UPF3B enable fault-tolerant activation of nonsense-mediated mRNA decay. *EMBO J*.. (2022) 41(10):e109191. doi: 10.15252/embj.2021109191

31. Wallmeroth D, Lackmann JW, Kueckelmann S, Altmuller J, Dieterich C, Boehm V, et al. Human UPF3A and UPF3B enable fault-tolerant activation of nonsense-mediated mRNA decay. *EMBO J*. (2022) 41:e109191. doi: 10.15252/embj.2021109191

32. Villate O, Turatsinze JV, Mascali LG, Grieco FA, Nogueira TC, Cunha DA, et al. Nova1 is a master regulator of alternative splicing in pancreatic beta cells. *Nucleic Acids Res*. (2014) 42:11818–30. doi: 10.1093/nar/gku861

33. Juan-Mateu J, Rech TH, Villate O, Lizarraga-Mollinedo E, Wendt A, Turatsinze JV, et al. Neuron-enriched RNA-binding proteins regulate pancreatic beta cell function and survival. *J Biol Chem*. (2017) 292:3466–80. doi: 10.1074/jbc.M116.748335

34. Aartsma-Rus A, van Ommen GJ. Antisense-mediated exon skipping: a versatile tool with therapeutic and research applications. *RNA*. (2007) 13:1609–24. doi: 10.1261/rna.653607

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Diagnosis, treatment, and management of rickets: a position statement from the Bone and Mineral Metabolism Group of the Italian Society of Pediatric Endocrinology and Diabetology

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Rickets results from impaired mineralization of growing bone due to alterations in calcium and phosphate homeostasis. Clinical signs of rickets are related to the age of the patient, the duration of the disease, and the underlying disorder. The most common signs of rickets are swelling of the wrists, knees or ankles, bowing of the legs (knock-knees, outward bowing, or both) and inability to walk. However, clinical features alone cannot differentiate between the various forms of rickets. Rickets includes a heterogeneous group of acquired and inherited diseases. Nutritional rickets is due to a deficiency of vitamin D, dietary calcium or phosphate. Mutations in genes responsible for vitamin D metabolism

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or function, the production or breakdown of fibroblast growth factor 23, renal phosphate regulation, or bone mineralization can lead to the hereditary form of rickets. This position paper reviews the relevant literature and presents the expertise of the Bone and Mineral Metabolism Group of the Italian Society of Pediatric Endocrinology and Diabetology (SIEDP). The aim of this document is to provide practical guidance to specialists and healthcare professionals on the main criteria for diagnosis, treatment, and management of patients with rickets. The various forms of rickets are discussed, and detailed references for the discussion of each form are provided. Algorithms to guide the diagnostic approach and recommendations to manage patients with rare forms of hereditary rickets are proposed.

KEYWORDS

hereditary rickets, management, non-hereditary rickets, nutritional rickets, treatment

1 Introduction

Rickets is a skeletal disease characterized by deficient mineralization of growth plates and bone matrix (osteomalacia) associated with low concentrations of calcium and/or phosphate in the blood. Rickets includes a heterogeneous group of acquired and inherited diseases. Nutritional rickets is the most common form of rickets globally. On the other hand, the development of molecular genetic techniques increased identification of the inherited forms of rickets. The primary method for diagnosing rickets involves examining the patient's medical history, conducting biochemical tests, and performing radiologic examinations. Genetic analyses are particularly important in cases where patients are suspected to have a genetic form of rickets despite the absence of a family history of the disease. Treatment and management of rickets should be targeted on the pathogenesis and both are strictly connected with the diagnosis.

The aim of this document is to provide practical guidance to specialists and healthcare professionals about the main criteria for diagnosis, treatment, and management of patients with rickets.

2 Methods

This position paper focuses on the main criteria for the diagnosis, treatment, and management of patients with rickets. Relevant studies were identified independently by three authors (G.B., S.M., and P.C.) from the Medline/PubMed database. This document is the result of a thorough discussion by the members of the Bone and Mineral Metabolism Group of the Italian Society of Pediatric Endocrinology and Diabetology (SIEDP) to standardize the procedures based on the relevant literature and on the expertise of the panel members. We did not grade the strength of our recommendations, but much information reported in this paper was obtained from studies that used this method.

The aim of the document was not to provide a literature review on rickets or to discuss in detail the various form of rickets. The intention was to provide stakeholders with selected data and suggest a correct approach for an early diagnosis of rickets. Some algorithms to facilitate the diagnosis are also presented. Moreover, some important aspects of the management of patients with different forms of rickets are proposed. This position paper presents a therapeutic approach to the various forms of rickets in light of recent guidelines, and a specific treatment for patients with X-linked hypophosphatemic rickets (XLH).

3 Classification of rickets

Rickets may be classified in various forms based on the primary biochemical alteration, such as hypocalcemia or hypophosphatemia, or subdivided into two main groups, such as nutritional and hereditary forms. Moreover, some rare forms of rickets may be associated with direct bone damage due to acquired or inherited disorders.

In this position paper, the various forms of rickets are subdivided into four major categories based on the main pathogenesis, such as nutritional, hereditary, non-hereditary hypophosphatemic, and other forms (Figure 1).

3.1 Nutritional rickets

Nutritional rickets is the most common form of rickets in developing countries, and it is frequently diagnosed in western countries among immigrant children, mainly from Africa and eastern Europe. Although it can rarely be caused by a phosphate-deficient diet, nutritional rickets is commonly the result of vitamin

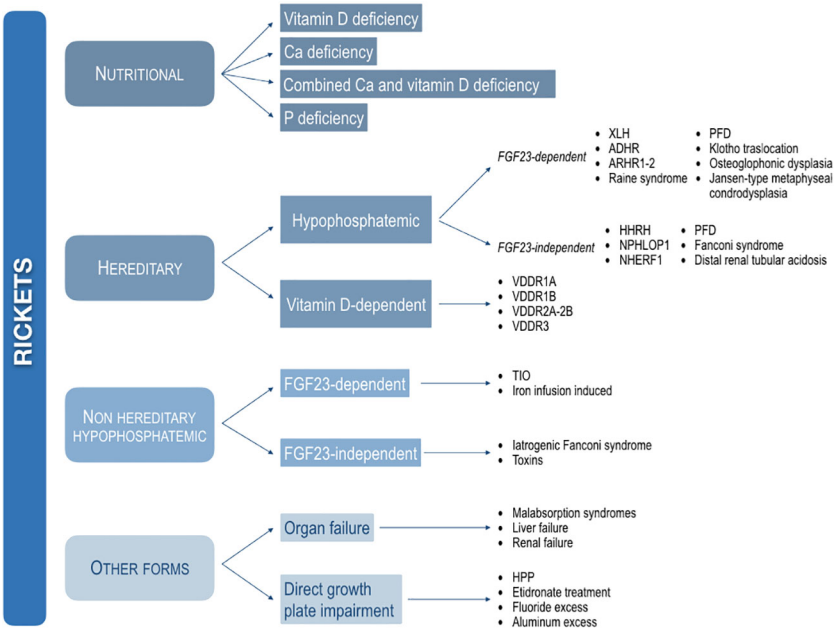

FIGURE 1
Classification of the main forms of rickets based on the pathogenesis. XLH, X-linked hypophosphatemic rickets; ADHR, autosomal dominant hypophosphatemic rickets; ARHR1, autosomal recessive hypophosphatemic rickets type 1; ARHR2, autosomal recessive hypophosphatemic rickets type 2; PFD, polyostotic fibrous dysplasia; HHRH, hypophosphatemic rickets with hypercalciuria; NPHLPO1, nephrolithiasis/osteoporosis, hypophosphatemic, 1. NHERF1, nephrolithiasis/osteoporosis, hypophosphatemic, 2; VDDR1A, vitamin D-dependent rickets type 1A; VDDR1B, vitamin D-dependent rickets type 1B; VDDR2A, vitamin D-dependent rickets type 2A; VDDR2B, vitamin D-dependent rickets type 2B; VDDR3, vitamin D-dependent rickets type 3; TIO, Tumor-induced osteomalacia; HPP, hypophosphatasia.

D deficiency due to insufficient UV exposure and/or insufficient dietary calcium intake, or a combination of both. This is particularly worrisome as nutritional rickets can profoundly affect the well-being of infants, children, and adolescents, with consequences that can persist into adulthood (1, 2).

In regions where calcium consumption is typically scant, with limited or absent dairy intake, dietary calcium deficiency stands as the primary culprit behind nutritional rickets among children beyond infancy (1, 3).

Dietary phosphate deficiency is a rare cause of nutritional rickets, but it is infrequent, because phosphate is abundant in natural foods. However, extreme starvation, a diet of elemental or hypoallergenic formula or parenteral nutrition, gastrointestinal surgery or short bowel syndrome, overuse of phosphate binders, or overly restrictive phosphate intake in managing chronic kidney disease may result in low phosphate intake (2, 4). Moreover, very low birthweight preterm infants are at high risk of developing nutritional rickets due to phosphate deficiency especially if they are breast-fed without phosphate supplements (2, 5).

3.2 Hereditary rickets

Hereditary rickets, caused by genetic mutations, may be subdivided into two main groups of disorders based on the association with impaired phosphate metabolism (hypophosphatemic rickets) or impaired vitamin D metabolism (vitamin D-dependent rickets) (Figure 1).

3.2.1 Hypophosphatemic rickets

Hypophosphatemic rickets include some disorders that may be associated with overproduction of fibroblast growth factor 23 (FGF23), namely FGF23-dependent forms, and disorders associated with a primary renal defect with normal concentrations of circulating FGF23, namely FGF23-independent forms (Figure 1).

3.2.1.1 FGF23-dependent hypophosphatemic rickets

X-linked hypophosphatemic rickets (XLH) is the commonest inherited form of rickets (6). Its prevalence is 1:20,000-60,000 (7, 8). XLH is due to mutation of *PHEX* gene encoding for phosphate regulating endopeptidase homolog X-linked, that regulates the expression of FGF23. The excessive production of FGF23 reduces tubular phosphate reabsorption and 1α-hydroxylase activity and stimulates renal 24-hydroxylase activity. Hypophosphatemia and a low or inappropriately normal serum 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) concentration in the setting of hypophosphatemia are the main biochemical findings of the patients with XLH (2, 9). Several other genes, such as *FGF23*, *DMP1*, and *ENPP1*, have a role in the synthesis, signaling, and regulation of FGF23. Mutations in these genes give rise to other rare variants of hypophosphatemic rickets, including autosomal dominant hypophosphatemic rickets (ADHR), autosomal recessive hypophosphatemic rickets type 1 (ARHR1) and autosomal recessive hypophosphatemic rickets type 2 (ARHR2), collectively constituting less than 20% of the cases (10).

Other rare forms of FGF23-dependent hypophosphatemic rickets include Raine syndrome due to mutation in the *FAM20C* gene (11), polyostotic fibrous dysplasia (PFD) due to gain-of-

function mutation of the *GNAS* gene (12), hypophosphatemic rickets and hyperparathyroidism due to translocation of the *KLOTHO* promoter (13), osteoglophonic dysplasia caused by dominant activation variants in *FGFR1* (14), opsismodysplasia (15), and Jansen-type metaphyseal chondrodysplasia due to mutations in the parathyroid hormone 1 receptor gene (16, 17). Further details are reported by Ackah et al. (14) and Shore (18).

3.2.1.2 FGF23-independent hypophosphatemic rickets

These disorders include some forms of hypophosphatemic rickets due to mutations in *SLC34A3* encoding for NaPi2C (hypophosphatemic rickets with hypercalciuria, HHRH), *SLC34A1* gene encoding for NaPi2a (nephrolithiasis/osteoporosis, hypophosphatemic 1, NPHLOP1), or mutation in *NHERF1* gene (nephrolithiasis/osteoporosis, hypophosphatemic, 2) (19, 20). Patients affected by Fanconi syndrome or distal renal tubular acidosis may develop hypophosphatemic rickets (2).

3.2.2 Vitamin D-dependent rickets

Vitamin D-dependent rickets are caused by mutations affecting the enzymes involved in the activation or degradation of vitamin D or the action/expression of the vitamin D receptor (VDR). Vitamin D-dependent rickets type 1A (VDDR1A) is due to impaired synthesis of $1,25(OH)_2D$ caused by a mutation in the enzyme (*CYP27B1*) encoding for renal 1α-hydroxylase. Vitamin D-dependent rickets type 1B (VDDR1B) is due to an impaired synthesis of 25-hydroxyvitamin D (25(OH)D) caused by a mutation in the enzyme (*CYP2R1*) encoding for hepatic 25-hydroxylase. Vitamin D-dependent rickets type 2 (VDDR2) is the result of impaired signaling of the VDR due to mutations in the *VDR* gene (VDDR2A) or the presence of a nuclear ribonucleoprotein that interferes with the VDR-DNA interaction (VDDR2B) (21). Vitamin D-dependent rickets type 3 (VDDR3) is due to increased inactivation of $1,25(OH)_2D$ caused by a gain-of-function mutation in a gene encoding a vitamin D-degrading enzyme (*CYP3A4*) (21, 22). The most striking biochemical sign of these forms of rickets is severe hypocalcemia with secondary hyperparathyroidism.

3.3 Non-hereditary hypophosphatemic rickets

This group of hypophosphatemic rickets includes some heterogeneous acquired disorders causing phosphate wasting. They may be associated with increased production of FGF23 or iatrogenic causes.

3.3.1 Acquired FGF23-dependent hypophosphatemic rickets

Tumor-induced osteomalacia (TIO) is due to the overproduction of FGF23 and other phosphaturic factors by benign slow-growing mesenchymal tumors (23, 24). TIO has rarely been described in children (25–30).

Iron infusion-induced reversible hypophosphatemic osteomalacia may occur in patients receiving intravenous iron preparations, especially with iron carboxymaltose or iron polymaltose, for treatment of anemia when oral iron is either ineffective or contraindicated (31). Iron infusion acutely impairs FGF23 cleavage, triggering transient increase in intact FGF23 and hypophosphatemia in association with an inappropriately low concentration of $1,25(OH)_2D$, similar to genetic diseases of primary FGF23 excess (14, 31).

3.3.2 Acquired FGF23-independent hypophosphatemic rickets

Iatrogenic renal Fanconi syndromes with hypophosphatemia may be caused by some drugs, including valproate, cisplatin, ifosfamide, gentamycin, and excessive use of phosphate binders (32, 33) and by some toxins, such as heavy metals antibiotics, or antiretroviral and anticancer medications (14).

3.4 Other causes of rickets

Rickets may be a consequence of congenital or acquired chronic disorders affecting the organs involved in the activation of vitamin D. Liver failure and renal failure are the main causes of impaired vitamin D metabolism. Moreover, malabsorption syndromes, i.e., cystic fibrosis, inflammatory bowel diseases, coeliac disease, or extensive surgical intestinal resection, may affect the intestinal absorption of vitamin D. These heterogeneous forms of rickets are also associated with various degrees of reduced bone mineral density that may be assessed by dual energy X-ray absorptiometry (DXA) (2, 34, 35).

Some rare forms of rickets are caused by direct impairment of the process of mineralization at the growth plate. Protracted etidronate disodium (ethane 1-hydroxy-1, 1-diphosphonate) administration in patients with generalized arterial calcification has been associated with radiologic signs of rickets likely due to impaired growth plate mineralization (36, 37). Patients with the infantile form of hereditary hypophosphatasia (HPP) show radiological lesions that may resemble rickets. A poorly mineralized ribcage may be associated with respiratory insufficiency (38, 39).

A rare form of hypophosphatemic rickets with hypocalciuria has been reported after long-term treatment with aluminum-containing antacids (40, 41).

Furthermore, a particular form of rickets was observed in Indian children exposed to a high intake of endemic fluoride in the drinking water since their birth. The association with calcium deficiency was a trigger for the development of fluoride toxicity in bone. In calcium-deficient children, the toxic effects of fluoride manifested even at marginally high (> 2.5 mg/day) exposures to fluoride (42).

4 Clinical approach to rickets

Medical history, physical examination, and the biochemical results are the main findings to diagnose a form of rickets. The

diagnosis of rickets should be confirmed by radiologic examination. The administration of vitamin D supplements, the evidence of relatives affected by rickets, the estimation of dietary calcium intake, and data on gestation and birth should be accurately investigated. Some clinical signs of rickets are more evident based on the age of the patient. Craniotabes (softening or thinning of the skull bones) may be the first sign of rickets in infants; it is detected by an inward collapse when applying pressure to the skull, typically followed by a snapping back after removing the pressure (43). In infants, other early signs of rickets are delayed fontanelle closure and frontal bossing (43, 44). In older children, swelling of the wrists, knees, or ankles and deformation of the legs such as knock-knees (genu valgum), or outward bowing (genu varum) with inability to walk are the main features (2, 44). Other skeletal signs of rickets are rachitic rosary, caused by swelling of the costochondral joints of the ribs, deformity of the soft rib cage, and bone pain (2, 44). Several non-osseous features may be associated with rickets: failure to thrive, delayed motor development, convulsions (due to hypocalcemia), muscular hypotonia, dilated cardiomyopathy, anemia, lethargy, irritability, delayed tooth eruption with altered enamel, and predisposition to respiratory infections (1, 43–48). HPP is characterized by diverse phenotypes that contribute to the diagnostic difficulties. In particular, neurological and dental involvements advocate for a multidisciplinary approach in the diagnosis of HPP (49).

Physical examination cannot differentiate children with active rickets from children recovering from rickets, and between the various forms of rickets (43). In addition, if abnormal bone features are detected clinically, the disease is already well established. Relying only on clinical signs may lead to overestimation of the prevalence of active rickets and lead to unnecessary treatment. The main skeletal signs of rickets are summarized in Table 1.

5 Biochemical features of the various forms of rickets

Serum calcium, phosphate, alkaline phosphatase, parathyroid hormone (PTH), and vitamin D metabolites are crucial biochemical parameters for the diagnosis of the various forms of rickets. Increased alkaline phosphatase activity is evident in all forms of rickets, except HPP (2, 4). Serum concentrations of phosphate and alkaline phosphatase activity should be interpreted according to appropriate age-specific reference values (49, 50).

Hypocalcemia is a main biochemical finding in patients with severe nutritional vitamin D deficiency rickets and in patients with vitamin D-dependent rickets. Hypophosphatemia (with normal serum calcium concentrations) is a specific feature in patients with hypophosphatemic rickets. However, hypophosphatemia may be also evident (usually associated with hypocalcemia) in patients with severe nutritional vitamin D deficiency rickets as a consequence of secondary hyperparathyroidism (2, 4, 35). It has been demonstrated that hypophosphatemia is the common denominator of all rickets affecting the process of apoptosis in the hypertrophic cells in the growth plate. In the absence of apoptosis, the hypertrophic cells accumulate in the growth plate and form the rachitic bone (51).

Renal tubular reabsorption of phosphate can be assessed by calculating the ratio of the tubular maximum reabsorption of phosphate to the glomerular filtration rate (TmP/GFR) (52). A reduced TmP/GFR ratio in patients with nutritional vitamin D deficiency rickets or vitamin D-dependent rickets reflects secondary hyperparathyroidism (2). The reduced TmP/GFR ratio found in patients with hypophosphatemic rickets is due to an overproduction or reduced degradation of fibroblast growth factor 23 (FGF23) (4, 20, 53).

Hypercalciuria may be an important diagnostic criterion in patients with HHRH (2, 4, 20, 35).

Secondary hyperparathyroidism is a major biochemical feature in patients with hypocalcemic forms of rickets. Serum PTH concentrations are normal or only slightly increased in patients with hypophosphatemic rickets, except in rare instances, such as in patients with HHRH who showed low normal o reduced serum PTH concentrations (2, 4, 20, 53).

There is strong evidence that nutritional vitamin D deficiency rickets develops with serum 25(OH)D concentrations <30 nmol/L (<12 ng/mL) (1), but it may also be associated with serum 25(OH)D concentrations >30 nmol/L (>12 ng/mL) (1, 54, 55). Furthermore, most children with vitamin D deficiency are asymptomatic (2, 56–58), and a normal calcium intake could be able to maintain bone

TABLE 1 Main skeletal and dental-periodontal signs in patients with rickets.

Cranium	Thorax and pelvis	Limbs	Total body and spine	Teeth and periodontium
• Frontal bossing • Craniosynostosis • Scaphocephaly • Occipital "bullet deformity" • Delayed anterior fontanel closure • Craniotabes[a] • Mid facial hypoplasia[b]	• Costo-chondral junction enlargement (rachitic rosary)[a] • Harrison sulcus[a] • Costal pathological fractures[a] • Pigeon chest • Chest wall asymmetry • Depressed ribs • Narrowed pelvic outlet	• Widened wrists, knees, and ankles • Genu-varum • Genu-valgum • Combined genu-varum/valgum • Short humerus[b] • Short femur[b] • Tibial torsion[c] • Coxa-vara	• Stunted growth • "Taylorwise" posture[a] • Disproportionate short stature (short limbs)[b] • Spinal curvature • Kyphosis	• Multiple dental decay[a] • Dyschromic enamel • Enamel hypoplasia • Delayed dentition • Abscesses with gingival fistulae[d]

[a]Mainly in patients with nutritional vitamin D deficiency rickets or vitamin D-dependent rickets; [b]mainly in patients with XLH; [c]intoeing or extoeing; [d]typical of patients with XLH: mainly in incisors and canines, without evidence of trauma or dental decay.

integrity (1). Some evidence suggested that a 25(OH)D concentration of 30 - 34 nmol/L (12 - 13.6 ng/mL) may be the critical cutoff value below which nutritional vitamin D deficiency rickets is more likely to occur (1, 59). A recent systematic review and multi-level meta-analyses by odds, sensitivities and specificities for nutritional rickets at different serum 25(OH)D thresholds suggested a minimal risk threshold of around 28 nmol/L (11 ng/ml) for children with adequate calcium intakes and 40 nmol/L (16 ng/ml) for children with low calcium intakes (60). Indeed, the total concentration of 25(OH)D and its circulating components may be influenced by factors such as the severity and duration of reduced 25(OH)D levels, habitual intake and bioavailability of dietary calcium, the rate of bone growth and mineralization, as well as genetic polymorphisms (2, 55, 61).

Reduced serum 25(OH)D concentrations are observed in patients with vitamin D hydroxylation-deficient rickets type 1B (62) and VDDR3 (22); conversely, patients with VDDR1A and VDDR2A or VDDR2B have normal or increased serum 25(OH)D concentrations (21).

Serum $1,25(OH)_2D$ concentrations vary with the time of diagnosis in nutritional vitamin D deficiency rickets. They are generally increased at the early stages of the disease to compensate for hypocalcemia and secondary hyperparathyroidism, whereas they are decreased at the late stages of the disease due to substrate depletion (48, 51). Therefore, measurement of $1,25(OH)_2D$ is not useful in patients with nutritional vitamin D deficiency rickets. In patients with hypophosphatemic rickets, serum $1,25(OH)_2D$ concentrations may be reduced, but in most patients, they are inappropriately normal in the setting of hypophosphatemia (2, 4, 35, 53). Serum $1,25(OH)_2D$ concentrations are increased in patients with VDDR2A, VDDR2B, nutritional calcium deficiency rickets, X-linked recessive hypophosphatemic rickets, HHRH or NPHLPO1 (2, 4, 20, 35).

FGF23 concentrations are increased in FGF23-dependent hypophosphatemic rickets (2, 4, 20, 35, 50); therefore, these conditions can be distinguished from FGF23-independent hypophosphatemic disorders by measuring the FGF23 concentration. Hypophosphatemia associated with intact FGF23 concentration greater than 40 pg/ml may be a crucial marker for the early diagnosis of XLH in pediatric patients (50). Assessment of FGF23 is also indicated in the diagnosis of patients with suspected TIO. Furthermore, FGF23 is the most critical biochemical marker for distinguishing nutritional vitamin D deficiency rickets from hypophosphatemic rickets, mainly in patients with an overlap of serum 25(OH)D concentrations (63). Moreover, increased FGF23 concentration is found in patients with calcium deficiency rickets (64).

Hypercalcemia and hyperphosphatemia, in addition to reduced alkaline phosphatase activity, are useful parameters for the differential diagnosis of the various forms of rickets (38, 39).

Biochemical features of the main forms of rickets are shown in Table 2.

6 Radiological assessment of rickets: diagnosis and follow-up

Plain radiography is the primary imaging tool for the assessment of rickets; the earliest signs are osteopenia and cortical thinning of long bones (48, 65). The initial assessment of a child suspected of having rickets should involve radiographs of skeletal sites where bones are undergoing the most rapid growth. These sites may differ depending on the child's age.

TABLE 2 Biochemical findings in patients with different types of rickets or rickets-like disorders.

Disorder	sCa	sP	ALP	PTH	25 (OH)D	1,25 $(OH)_2D$	FGF23	uCa	Tmp
Nutritional rickets									
Nutritional vitamin D deficiency rickets	N, ↓	↓	↑	↑	↓	↑, N, ↓	N	↓	↓
Nutritional calcium deficiency rickets	N, ↓	↓	↑	↑	N, ↓	↑	N, ↑	↓	↓
Nutritional phosphate deficiency	N, ↑	↓	↑	N, ↓	N	N, ↑	N, ↓	N, ↑[a]	N, ↓[b]
Hereditary FGF23-dependent hypophosphatemia									
XLH, ADHR, ARHR1, ARHR2	N	↓	↑	N, ↑[c]	N	N[d], ↓	N, ↑	N, ↓	↓
Raine syndrome	N, ↓[e]	N, ↓ [e]	↑	N, ↑	N	N, ↓	N, ↑	N	↓
PFD	N, ↑	N, ↓	↑	N, ↑	N	N, ↓	N, ↑	N	N, ↓
Hypophosphatemic rickets and hyperparathyroidism	N, ↑	↓	↑	↑	N	N[d], ↓	↑	N, ↑	↓
Osteoglophonic dysplasia	N	↓	N, ↑	N	N	N[d], ↓	N, ↑	N	↓
Opsismodysplasia	N	↓	N, ↑	N	N	N[d], ↓	N, ↑	N	↓
Jansen-type metaphyseal chondrodysplasia	N, ↑	↓	↑	N, ↓	N	N, ↑	↑	N, ↑	↓
Hereditary FGF23-independent hypophosphatemia									
HHRH	N	↓	↑	N, ↓	N	N, ↑	N, ↓	↑	↓

(Continued)

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TABLE 2 Continued

Disorder	sCa	sP	ALP	PTH	25 (OH)D	1,25 $(OH)_2D$	FGF23	uCa	Tmp
Hereditary FGF23-independent hypophosphatemia									
NPHLPO1	N	↓	↑	variable	N	↑	↓	↑	↓
NHERF1	N	↓	?	N	N	N, ↑	N	↑	↓
X-linked recessive hypophosphatemic rickets	N	↓	↑	variable	N	↑	?	N, ↑	↓
Fanconi syndrome or distal renal tubular acidosis	↑, N, ↓	N, ↓	↑	N, ↑[e]	N	N, ↓	N, ↑[f]	N, ↑	N, ↓
Linear sebaceous nevus syndrome	N, ↓	↓	↑	N, ↑[c]	N	N[d], ↓	N, ↑	N, ↓	↓
Vitamin D-dependent rickets									
VDDR1A	↓	↓	↑	↑	N	↓	N, ↓	↓	↓
VDDR1B	↓	↓	↑	↑	↓	↓	N	↓	↓
VDDR2A-2B	↓	↓	↑	↑	N	↑	N, ↓	↓	↓
VDDR3	↓	↓	↑	↑	↓	↓	?	↓	↓
Acquired FGF23-dependent hypophosphatemia									
Tumor-induced osteomalacia	N, ↓	↓	↑	N, ↑[c]	N	N[d], ↓	N, ↑	N, ↓	↓
Ferric carboxymaltose i.v. infusion	↓	↓	↑	↑	N	↓	↑[f], ↓[g]	?	↓
Acquired FGF23-independent hypophosphatemia									
Iatrogenic proximal Fanconi syndromes	N	↓	↑	variable	N	↑	↓	variable	↓
Other causes of rickets									
Malabsorption, liver insufficiency/failure, renal insufficiency/failure	N, ↓	↑, N, ↓	↑	N, ↑	N, ↓	variable	↑[h]	variable	↓[i]
Etidronate treatment	N	↓	↑	N	N	↑	?	N	↓
HPP	N, ↑	↑	↓	N, ↓	N	N, ↓	N	↑	↑

sCa, serum calcium; sP, serum phosphate; ALP, alkaline phosphatase; PTH, parathyroid hormone; 25(OH)D, 25-hydroxyvitamin D; 1,25$(OH)_2D$, 1,25-dihydroxyvitamin D; FGF23, fibroblast growth factor 23; uCa, urinary calcium, TmP, renal tubular reabsorption of phosphate.
XLH, X-linked hypophosphatemic rickets; ADHR, autosomal dominant hypophosphatemic rickets; ARHR1, autosomal recessive hypophosphatemic rickets type 1; ARHR2, autosomal recessive hypophosphatemic rickets type 2; PFD, polyostotic fibrous dysplasia; HHRH, hypophosphatemic rickets with hypercalciuria; NPHLPO1, nephrolithiasis/osteoporosis, hypophosphatemic, 1. NHERF1, nephrolithiasis/osteoporosis, hypophosphatemic, 2; VDDR1A, vitamin D-dependent rickets type 1A; VDDR1B, vitamin D-dependent rickets type 1B; VDDR2A, vitamin D-dependent rickets type 2A; VDDR2B, vitamin D-dependent rickets type 2B; VDDR3, vitamin D-dependent rickets type 3; HPP, hypophosphatasia.
↑: increased concentration; ↓: decreased concentration; N: normal concentration;?: not known.
Nutrional phosphate deficiency: biochemical findings are referred to dietary phosphate deficiency in very low birthweight infants.
[a]Normal after restoration of P intake, but falsely increased before restoration.
[b]Normal after restoration of P intake, but falsely reduced before restoration. Urinary phosphate excretion is very low or undetectable before restoration of P intake.
[c]It may be moderately above the upper limit of normal.
[d]decreased relative to the serum phosphate concentration.
[e]depending on the stage of chronic kidney disease.
[f]Intact FGF23.
[g]C-terminal FGF23.
[h]in patients with renal insufficiency/failure.
[i]in patients with secondary hyperparathyroidism or excessive production of FGF23 due to renal insufficiency/failure.

A radiological examination of the chest may be useful to assess rickets in neonates, mainly in preterm infants, and in infants in the first year of life (65, 66). Insufficient mineralization of the metaphyseal-physeal region can be observed in the proximal humerus and anterior rib ends (rachitic rosary). In most children, radiological signs of rickets are evident at the wrists and knees, where rapid growth occurs. The distal ulna is the best site to demonstrate early changes of rickets, mostly in young children (67). The classical rachitic changes are the widening and irregularity of all the physes, with metaphyseal widening, splaying, cupping, and fraying. Moreover, a coarse trabecular pattern may be observed in the metaphysis (48, 65). Epiphyseal ossification centers become blurred or apparent (65). Cranial deformities and bowing of the legs are the typical signs of osteomalacia due to unmineralized osteoid (66).

Pathologic fractures (including rib fractures) are rare except in very preterm babies and are very uncommon beyond age 6 months corrected postnatal age (66). The occurrence of fractures is usually evident in children with a severe form of rickets (68). Fractures of long bones may occur occasionally in children affected by calciopenic and phosphopenic (excluding XLH) forms of rickets in association with radiographic signs of osteopenia and the evidence of typical metaphyseal rachitic lesions (2).

Moreover, by radiologic examination is possible to estimate the severity of rickets by using the Rickets Severity Score (RSS). This score is determined based on the degree of metaphyseal fraying, concavity, and the proportion of the growth plate affected at the wrists, knees, and ankles (69, 70). The RSS employs a 10-point scale, with 10 indicating the utmost severity of rickets, and 0 denoting the absence of any radiographic alterations. The radiographic response after treatment of nutritional vitamin D deficiency rickets (69) and XLH (70) can be estimated by the RSS, which correlates with serum alkaline phosphatase activity.

The Radiographic Global Impression of Change (RGI-C) represents a complementary assessment of RSS. A change score is assigned based on differences in the appearance of rickets on pairs of radiographs compared side by side. The RGI-C has been validated to evaluate skeletal changes in patients with HPP (71). Moreover, Lim et al. (72) demonstrated that RGI-C is a reliable, valid, and sensitive tool in patients with XLH, and complementary to the RSS.

Radiologic examination is also useful to confirm the diagnosis of rickets suspected by clinical signs and biochemical data, but it does not indicate the pathogenesis, because the radiological signs of rickets are similar in patients with nutritional rickets, hypophosphatemic rickets, and vitamin D-dependent rickets (Figure 2).

Finally, radiological examination is useful to examine the effect of treatment with vitamin D in patients with nutritional vitamin D deficiency rickets showing the appearance of the zone of provisional calcification at the ends of the metaphyses that is usually seen within 3-4 weeks of treatment (67).

7 Algorithms for the diagnosis of rickets

Figure 3 shows a management algorithm based on the biochemical parameters for the evaluation of patients with rickets confirmed by the evidence of clinical signs and the typical radiologic lesions of rickets and associated with increased serum alkaline phosphatase activity. The algorithm is primarily based on the pathogenesis of hypocalcemia and hypophosphatemia; serum concentrations of calcium, phosphate, and PTH represent the key biochemical parameters to differentiate hypocalcemic from hypophosphatemic form of rickets. Hypocalcemic forms of rickets are usually characterized by hypocalcemia, a low or normal serum phosphate concentration, and increased PTH values; whereas, hypophosphatemia with normocalcemia and normal PTH concentrations are the main biochemical signs of most of the hypophosphatemic forms of rickets.

The measurement of serum concentrations of vitamin D metabolites may indicate the pathogenesis of rickets in patients with hypocalcemic forms. Reduced serum values of 25(OH)D and $1,25(OH)_2D$ suggest the diagnosis of VDDR1B or VDDR3, whereas normal or increased serum 25(OH)D values associated with low $1,25(OH)_2D$ may be diagnostic for VDDR1A. A normal or increased serum 25(OH)D concentration combined with a normal or increased serum $1,25(OH)_2D$ concentration may characterize two conditions, nutritional calcium deficiency or VDDR2.

The measurement of FGF23 may be useful to identify the FGF23-mediated from the non-FGF23-mediated hypophosphatemic disorders. High FGF23 concentrations are evident in both hereditary and acquired forms of hypophosphatemic rickets such as TIO. Hypophosphatemia associated with normal or low FGF23 concentrations suggests the diagnosis of a form of Fanconi syndrome or HHRH. Patients with HHRH usually show hypercalciuria. Hypophosphatemia with high TmP/GFR values may indicate the diagnosis of nutritional phosphate deficiency rickets.

8 Genetics

Whenever the clinical, biochemical, and radiologic evidence indicates that the cause of rickets might be of genetic origin, specific molecular testing is necessary. Table 3 summarizes the genes involved in the genesis of genetically determined rickets or hypophosphatemia.

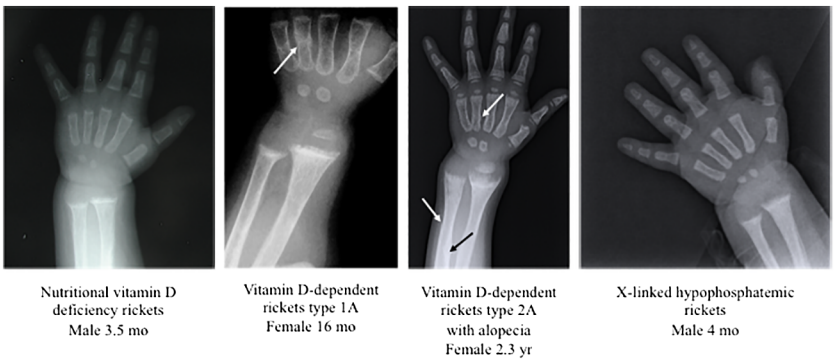

FIGURE 2
Radiographic features at the wrist and at the hand in patients with nutritional vitamin D deficiency rickets and some forms of hereditary rickets. In patients with hereditary rickets, the diagnosis was confirmed by genetic analyses. All patients showed widening and fraying of the epiphyseal plate and metaphyseal concavity of the ulna. The white and black arrows showed fractures.

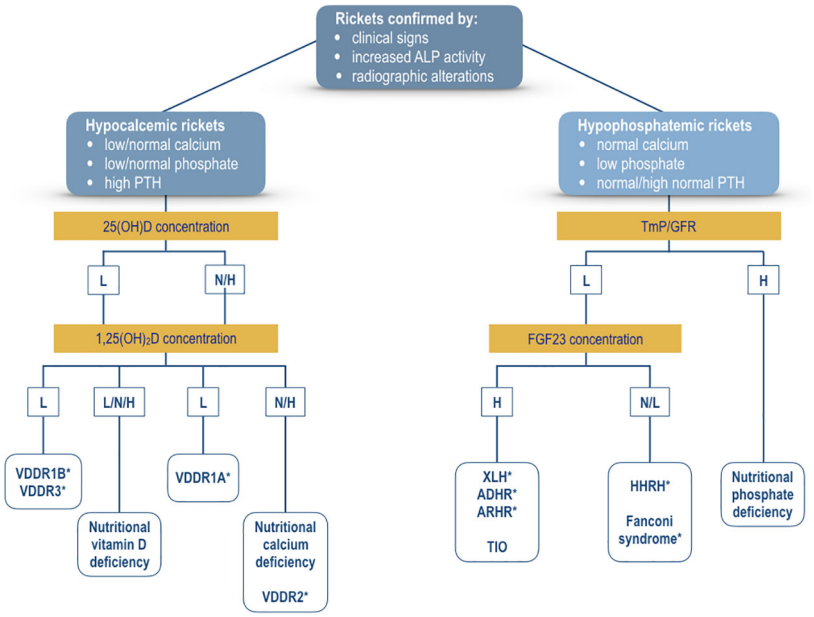

FIGURE 3
Algorithm for the evaluation of a patient with confirmed rickets by clinical signs, increased alkaline phosphatase activity, and radiographic lesions of rickets. The primary differential diagnosis is based on the presence of hypocalcemia (hypocalcemic rickets) or hypophosphatemia (hypophosphatemic rickets). Measurement of serum concentrations of 25(OH)D and 1,25$(OH)_2$D may indicate the biochemical diagnosis of the various forms of hypocalcemic rickets. Measurement of TmP/GFR values represents the first step for the selection of the patients with hypophosphatemic rickets. Thereafter, measurement of FGF23 concentration may suggest the biochemical diagnosis of the various forms of hypophosphatemic rickets. For additional detais see text. XLH, X-linked hypophosphatemic rickets; ADHR, autosomal dominant hypophosphatemic rickets; ARHR1, autosomal recessive hypophosphatemic rickets type 1; ARHR2, autosomal recessive hypophosphatemic rickets type 2; HHRH, hypophosphatemic rickets with hypercalciuria; VDDR1A, vitamin D-dependent rickets type 1A; VDDR1B, vitamin D-dependent rickets type 1B; VDDR2A, vitamin D-dependent rickets type 2A; VDDR2B, vitamin D-dependent rickets type 2B; VDDR3, vitamin D-dependent rickets type 3; TIO, Tumor-induced osteomalacia. *Genetic testing is important to confirm the suspected diagnosis. L, low values; N, normal values; H, high values.

The analysis of the *PHEX* gene in the suspect of XLH should be performed, bearing in mind the extensive heterogeneity of the pathogenic variants. These have been found in all exons and in the non-coding regions. Numerous are the insertions and deletions that have also been reported (73, 74).

9 Treatment and management of nutritional rickets

9.1 Nutritional vitamin D deficiency rickets

Vitamin D supplements may be administered as daily therapy or as a single bolus; the suggested doses are reported in Table 4. Alternatively, a bolus of 100,000 IU every 20 days for 3-4 times may be effective in patients with poor compliance with the treatment (75). Administration of a single-day high-dose of vitamin D therapy (stoss therapy), with doses reaching up to 600,000 IU, has been proposed. However, hypercalcemia may occur (76, 77). Therefore, the use of high and repeated doses of vitamin D is not recommended. Daily treatment with vitamin D should be continued for 3-4 months (1, 2, 35, 48, 75, 78), but the duration of treatment should be individualized. Normalization of skeletal lesions is usually reached during this time. If radiographic recovery remains incomplete or serum biochemistry hasn't normalized, vitamin D treatment should be extended (79). Inadequate adherence to the treatment regimen may lead to failure or delayed healing of rickets. In this occurrence, vitamin D stoss therapy by a single intramuscular injection ranging from 50,000 IU for children aged 3 months and older, up to 300,000 IU for individuals over 12 years may be recommended (1).

Some studies showed that in increasing serum 25(OH)D concentration the daily administration of vitamin D_3 was similar to vitamin D_2, whereas, vitamin D_3 was more effective than vitamin D_2 when administered as bolus (80). Moreover, there is no indication for the use of vitamin D metabolites for the treatment of patients with nutritional vitamin D deficiency rickets, because they do not restore vitamin D status and hypercalcemia may occur (66, 67, 81).

Patients experiencing symptomatic hypocalcemia, such as tetany, convulsions, or dilated cardiomyopathy, should receive intravenous calcium gluconate until serum calcium levels are normalized (35, 75, 78). Once normocalcemia is achieved, transitioning to oral calcium supplementation is appropriate. A routine oral calcium supplementation of 500 mg/day, obtained through dietary intake or supplements, should be implemented concurrently with vitamin D, irrespective of age or weight. Duration of calcium supplementation is variable and is related to the timing of normalization of serum calcium concentration (1). Alternatively, 30-75 mg/kg/day elemental calcium in 3 divided doses may be administered over 2-4 weeks. Calcium supplements are important to avoid hypocalcemia as the concentration of PTH normalizes

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TABLE 3 Genes involved in the genesis of genetically determined rickets or hypophosphatemia.

Disorder	Gene	OMIMM #
XLH	*PHEX*	307800
ADHR	*FGF23*	193100
ARHR1	*DMP1*	241520
ARHR2	*ENPP1*	613312
Raine syndrome	*FAM20C*	259775
PFD	*GNAS*	174800
HHRH	*SLC34A3*	241530
Hypophosphatemic rickets and hyperparathyroidism	*13q13.1*	612089
Osteoglophonic dysplasia	*FGFR1*	166250
Opsismodysplasia	*INPPL1*	258480
Jansen-type metaphyseal chondrodysplasia	*PTH1R*	156400
NPHLOP1	*SLC34A1*	612286
NHERF1	*SLC9A3*	604990
X-linked recessive hypophosphatemic rickets	*CLCN5*	300554
VDDR1A	*CYP27B1*	264700
VDDR1B	*CYP2R1*	600081
VDDR2A	*VDR*	277440
VDDR2B	*VDR*	600785
VDDR3	*CYP3A4*	619073

XLH, X-linked hypophosphatemic rickets; ADHR, autosomal dominant hypophosphatemic rickets; ARHR1, autosomal recessive hypophosphatemic rickets type 1; ARHR2, autosomal recessive hypophosphatemic rickets type 2; PFD, polyostotic fibrous dysplasia; HHRH, hypophosphatemic rickets with hypercalciuria; NPHLPO1, nephrolithiasis/osteoporosis, hypophosphatemic, 1. NHERF1, nephrolithiasis/osteoporosis, hypophosphatemic, 2; VDDR1A, vitamin D-dependent rickets type 1A; VDDR1B, vitamin D-dependent rickets type 1B; VDDR2A, vitamin D-dependent rickets type 2A; VDDR2B, vitamin D-dependent rickets type 2B; VDDR3, vitamin D-dependent rickets type 3.

("hungry-bone" syndrome), particularly in patients treated with stoss therapy (48). Doses of intravenous and oral calcium supplements are reported in Table 5.

Monitoring of treatment with vitamin D and calcium supplements varies with the severity of rickets and response to therapy. Normalization of serum calcium and phosphate concentrations usually occurs within 3 weeks (67, 75), but it may also be evident after only 6-10 days of treatment (48). Serum PTH concentrations usually fall within the normal range as normocalcemia is restored (75, 81). Serum 25(OH)D concentrations increase rapidly and normal values may be reached after 4-6 weeks (75). Serum $1,25(OH)_2D$ concentrations increase rapidly with treatment and remain elevated for up to 10 weeks (81). Alkaline phosphatase activity declines progressively but it may remain increased for several months (3-6 months) depending on the severity of the vitamin D deficiency (48, 82). Moreover, alkaline phosphatase activity is associated with the recovery of skeletal rachitic lesions, suggesting that it is a reliable and economic biochemical marker for monitoring the effectiveness of treatment in the clinical setting (82).

TABLE 4 Vitamin D treatment in patients with nutritional vitamin D deficiency rickets.

Age	Daily dose for 3 months, IU	Single dose, IU	Maintenance daily dose, IU
< 3 months	2000	–	400
3-12 months	2000	50,000	400
> 12 months to 12 years	3000-6000	150,000	600
> 12 years	6000	300,000	600

Response to treatment based on clinical, biochemical, and radiologic assessments, should be performed after 3 months of vitamin D administration; some patients may require further treatment. A daily calcium intake of at least 500 mg should be ensured, either as a dietary intake or supplements. For conversion from IU to µg, divide by 40.
Adapted from Munns et al. (1).

In order to prevent the resurgence of nutritional vitamin D deficiency rickets vitamin D supplementation should be continued. Following the resolution of rickets, at least 400 IU/day before the age of 12 months and 600 IU/day of vitamin D are recommended (1, 67, 78).

9.2 Nutritional calcium deficiency rickets

A randomized controlled study in children with nutritional calcium-deficiency rickets showed that radiographic healing of the rachitic lesions was obtained by daily administration of with 500 mg, 1000 mg, or 2000 mg of elemental calcium salts. However, daily supplementation with 1000 mg and 2000 mg was more effective than 500 mg. The treatment with 2000 mg was similar to 1000 mg of supplemental calcium (83). Therefore, it is recommended that 1000 mg of elemental calcium daily (subdivided into 2-3 doses) should be used in children with nutritional calcium deficiency rickets, until healing is complete; in some children, it may take more than 24 weeks (84). The rate of healing of rickets may be improved by

TABLE 5 Calcium treatment in patients with symptomatic or asymptomatic hypocalcemia associated with rickets.

Condition	Calcium salts	Doses, mg/kg[a] (mL)	Mode of administration
Symptomatic acute hypocalcemia	Gluconate 10%[b]	5-20 (0.5-2.0)	Intravenous slowly, over 10-15 min to avoid bradycardia[c]; diluted in 0.9% sodium chloride or 5% dextrose
Asymptomatic hypocalcemia or normocalcemia	Carbonate, citrate	30-75 5 years old: 500 mg/day[d] 10 years old: 1000 mg/day[d]	Oral route, divided into 2-3 doses daily

[a]As elemental calcium. Calcium carbonate is 40% elemental calcium; calcium citrate is 21% elemental calcium.
[b]One ampoule (10 mL) contains about 90 mg of elemental calcium.
[c]Electrocardiographic monitoring is strongly recommended.
[d]Suggested by Lambert AS and Linglart A (35).

vitamin D supplements (50,000 IU every 4 weeks) (84). Radiologic healing of the growth plate usually occurred after 3-6 months of calcium administration, even though the clinical signs of rickets required a longer time to resolve than biochemical and radiologic alterations (3).

9.3 Nutritional phosphate deficiency rickets

Management of dietary phosphate deficiency is based primarily on adequate dietary supplements. Oral or parenteral phosphate salts are usually administered in patients with impaired phosphate absorption after extensive gastrointestinal surgery, short bowel syndrome, or severe gastrointestinal disorders (78).

Biochemical parameters, including relative hypocalcemia or hypophosphatemia, are the main factors for the management of metabolic bone disease of prematurity. Measurement of PTH has a pivotal role for the treatment, because increased PTH occurs in calcium deficiency, while it is normal or reduced in phosphate deficiency (85, 86). Human milk fortifiers and special preterm formulas are usually indicated to improve the needs for growth in very low birthweight infants. Phosphate supplementation should be considered for serum phosphate concentrations <4 mg/dL (1 mmol/L), but it can be also indicated if values fall below 5.5 mg/dL (1.3 mmol/L), especially if associated with increased alkaline phosphatase activity, to improve bone mineralization and to prevent hypercalciuria (87). The recommended daily oral intake of calcium and phosphate varies between 150-220 mg/kg/day and 75-140 mg/kg/day through enteral feeds, respectively (87–91); an enteral calcium to phosphate intake ratio at 1.5:1 to 1.7:1 on mg-to-mg basis is proposed (86, 87). For parental nutrition, calcium 75-100 mg/kg/day and phosphate 50-80 mg/kg/day are recommended (85, 86, 90, 91).

In infants unable to tolerate human milk fortifier or preterm formula, elemental minerals may be added by oral route. Supplementation usually starts at 20 mg/kg/day of elemental calcium and 10-20 mg/kg/day of elemental phosphorus. It is increased, as tolerated, to a maximum of 70-80 mg/kg/day of elemental calcium and 40-50 mg/kg/day of elemental phosphorus (87, 91). Serum PTH concentrations and alkaline phosphatase activity should be measured every 1-2 weekly for adjusting phosphate and calcium to phosphate ratios (86).

Vitamin D supplements in preterm infants differ between United States and Europe recommendations. The European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) recommends a dose of 800-1000 IU vitamin D daily, whereas 200 IU/day is recommended in the United States for infants weighing less than 1500 g and 400 IU/day for infants weighing more than 1500 g (87, 88, 91, 92). A lower calcium intake may benefit from a higher vitamin D intake as suggested by ESPGHAN recommendations, whereas a higher calcium intake may be preferred in association with a lower vitamin D intake as indicated in the United States (91, 92).

10 Treatment and management of vitamin D-dependent rickets

10.1 VDDR1A

Conventional treatment for patients with VDDR1A is based on the association of a vitamin D active metabolite and calcium supplements. Patients must be treated lifelong with physiologic doses of $1,25(OH)_2D$ given twice daily due to its short half-life (21). Alternatively, alfacalcidol may be administered once a day due to its longer half-life. Alfacalcidol treatment by drop formulation may be useful in affected infants. The dosage of both calcitriol and alfacalcidol should be titrated according to serum calcium concentrations. The high calcium demands of the unmineralized skeleton occurring in the first months of treatment may require 2-5 times higher dosages of active vitamin D metabolites than during maintenance treatment (21, 93). The maintenance doses of active vitamin D metabolites and calcium supplements after normalization of serum calcium and PTH concentration are reported in Table 6.

Response to calcitriol or alfacalcidol is usually rapid, with healing of rickets and normalization of the biochemical parameters within 7-9 weeks (94). Long-term compliance with the treatment is important to maintain normal calcium and PTH concentrations. Serum concentrations of calcium, phosphate, and PTH, and urinary calcium excretion should be measured regularly during the maintenance treatment (at least every 4-6 months). However, time intervals may be reduced if hypocalcemia persists during the first weeks of treatment. Serum calcium concentration should be maintained in the low-normal range to keep PTH secretion below the upper limit of normal (95). Calcium excretion should be maintained below the normal limit for age and weight. Renal ultrasound examination should be performed every 1-2 years to monitor for renal calcification, and more frequently if there is evidence of hypercalciuria (21). However, based on personal expertise renal ultrasonography every 6-12 months may be useful for the early diagnosis of a mild form of nephrolithiasis.

10.2 VDDR1B

The best treatment for the patients with this form of rickets requires the association of calcifediol, as vitamin D has not/poor effect due to the altered hepatic conversion, and calcium supplements (Table 6). Serum 25(OH)D concentrations increased in response to a vitamin D bolus in heterozygous patients, although the achieved concentrations were lower than those found in controls. Homozygous patients with mutations in *CYP2R1* showed a very limited or no increase in serum 25(OH)D concentrations in response to the same vitamin D bolus (62, 96). Alternatively, other vitamin D active metabolites, such as calcitriol or alfacalcidol, may be effective in improving serum calcium and PTH concentrations.

TABLE 6 Maintenance treatment in patients with vitamin D-dependent rickets.

Drug	VDDR1A	VDDR1B	VDDR2	VDDR3
Vitamin D	NI	Heterozygous: 5,000-10,000 IU/day Homozygous: 600,000 every 3 months	LI	50,000/day
Calcifediol	NI	15-50 µg/day	20-200 µg/day[a]	?
Alfacalcidol	10-100 ng/kg/day 0.5-3 µg/day	0.5-3 µg/day	10-400 ng/kg/day[b] 5-60 µg/day[b]	?
Calcitriol	10-100 ng/kg/day 0.3-2 µg/day	0.3-2 µg/day	10-400 ng/kg/day[b] 5-60 µg/day[b]	?
Calcium salts (by oral route)	0.5-3 g/day	0.5-2 g/day	3-5 g/day 400-1400 mg/m^2/day[c]	?

NI, not indicated.
LI, little indicated.
[a]only some patients without alopecia may respond.
[b]some patients do not respond despite maximal doses.
[c]intravenous administration for many years.
Adapted and modified from Levine MA (21).

10.3 VDDR2A and VDDR2B

The success of the treatment of patients with VDDR2A varies according to the VDR mutation (97). It has been found that mutations in the ligand-binding domain are associated with variable resistance to 1,25 $(OH)_2D$; whereas, mutations in the DNA binding domain cause almost total 1,25$(OH)_2D$ resistance and alopecia (2). Patients with alopecia are usually less responsive to treatment in comparison with patients without alopecia (98). However, the response to treatment is highly variable and the doses of vitamin D metabolites and calcium salts should be individualized. About half of patients with alopecia are resistant even to the highest vitamin D metabolites doses. The other half require ten times higher vitamin D metabolites doses than patients without alopecia (21, 78).

Levine (21) suggested that maintenance therapy is based upon several factors: (a) some patients will respond to calciferols (vitamin D_2, vitamin D_3, or calcifediol), which are substrates for the generation of 1,25 $(OH)_2D$; (b) other patients may respond only to high doses of vitamin D metabolites (calcitriol or alfacalcidol) that possess 1α-hydroxylation; (c) a minority of patients will not respond to calciferols Some patients affected by severe forms of the disease required long-term intravenous calcium administration (given over 12 h, often during the night) for many months in order to achieve normocalcemia (21, 78, 99).

During and after puberty, the patients may develop an increased intestinal calcium absorption to concentrations that are even greater than those of normal individuals. Indeed, some patients maintain normocalcemia with modest oral calcium supplementation, and a small number of patients may have normocalcemia even without calcium supplements (21).

Management of patients with VDDR2B is similar to that indicated in patients with VDDR2A (21, 99).

10.4 VDDR3

Few data on the treatment of patients with VDDR3 are available. These patients may respond to vitamin D or vitamin D metabolites, but greater doses are required in comparison within patients with nutritional vitamin D deficiency rickets. Lifelong treatment with a high dose of vitamin D are needed to ensure clinical and biochemical remission (21, 22). Some suggestions for the treatment of these patients are reported in Table 6.

11 Treatment and management of hypophosphatemic rickets

11.1 FGF23-dependent hypophosphatemic rickets

Conventional treatment of patients with FGF23-dependent hypophosphatemic rickets, including XLH, ADHR, ARHR1, and ARHR2, consists of inorganic oral phosphate salts combined with vitamin D active metabolites, such as calcitriol or alfacalcidol. Most of the data on the use of conventional treatment in hypophosphatemic rickets comes from the management of patients with XLH. The recommended starting and maintenance doses of inorganic phosphate salts and vitamin D active metabolites in patients with hypophosphatemic rickets (2, 100–103) are summarized in Table 7. Conventional treatment may be associated with a transient increase in serum phosphate concentration only in some patients, without significant changes in TmP/GFR. The treatment started in early infancy improves outcomes even if it did not completely normalize skeletal abnormalities (2, 104–106). It is generally associated with slow improvement in the healing of rickets and with residual skeletal deformities in most patients. Moreover, it has been shown that patients with XLH treated with conventional treatment had decreased height gain by 1 year of age and remained below population norms thereafter (107). This suggests that XLH affects skeletal growth and bone mineralization from the earliest years of life and that conventional treatment may not improve the evolution of the disease.

Conventional treatment may be effective in improving dental and parodontal lesions (100, 108, 109) depending on the onset, compliance, and duration of therapy, even though some studies demonstrated a poor effect of the conventional treatment on oral phenotype (110, 111).

In patients with ADHR and ARHR1 the dosages of phosphate supplements and vitamin D active metabolites may vary according to the severity of the disease and the response to treatment. In patients with ADHR, it has been shown that iron deficiency affected the severity of the phenotype, and that iron repletion normalized previously increased FGF23 concentrations and improved serum phosphate concentration (112). Therefore, reduction or withdrawal of conventional treatment should be attempted only after the optimization of iron status by oral iron administration (3 to 6 mg/kg elemental Fe, max 200 mg/day for 3 months) (113, 114). Measurement of ferritin is suggested to assess the need for iron supplementation in patients with ADHR (114).

Inorganic oral phosphate salts and vitamin D active metabolites constitute the conventional treatment also for patients with ARHR2 (115–117). However, the fact that ENPP1 deficiency may also be associated with arterial, cardiac, and articular calcification, or may present as generalized arterial calcification (115, 118, 119), should be taken into consideration because these phenotypes preclude the administration of the conventional treatment; therefore, the assessment of carotid intima media thickness and cardiac ultrasonography are strongly recommended in patients with ARHR2 (78). However, recent studies in patients with ARHR2 indicated that conventional treatment did not result in increased calcification in patients with ENPP1 deficiency (120, 121). Furthermore, patients with biallelic *ENPP1* mutations may develop normocalcemic primary hyperparathyroidism, which may require partial parathyroidectomy (116).

In patients with a form of FGF23-dependent hypophosphatemic rickets, the benefit of the conventional treatment should be balanced with the potential risks of overtreatment (53). Compliance with the treatment is poor in many patients, mainly in infants, due to gastrointestinal symptoms, including diarrhea, bloody stools, and abdominal pain; compliance may improve by reducing the dose of oral phosphate salts (53). Some complications have been reported in patients with XLH during conventional treatment, including hypercalcemia, secondary/tertiary hyperparathyroidism, hypercalciuria, nephrocalcinosis, and nephrolithiasis (53, 100–103). Some recommendations for follow-up in patients with XLH receiving conventional treatment have been suggested (2, 53, 100–103, 122) (Table 8).

TABLE 7 Starting and maintenance doses of inorganic phosphate salts and active vitamin D analogs in patients with hypophosphatemic rickets.

	Newborns or before the development of clinical or radiological signs of rickets	Evidence of clinical or radiological signs of rickets
Starting doses	Alfacalcidol, 25-40 ng/kg/day (0.8-1 µg/day), once/day; inorganic phosphate salts, 20-40 mg/kg/day, 4 to 6 intakes/daily	Alfacalcidol, 40-80 ng/kg/day (1-1.5 µg/day), once/day calcitriol, 20-40 ng/kg/day, 2-3 times/day; inorganic phosphate salts, 40-60 mg/kg/day, 4 to 6 intakes/daily
Maintenance doses	Alfacalcidol, 25-40 ng/kg/day (1-2 µg/day), once/day; calcitriol, 20-30 ng/kg/day, 2-3 times/day; inorganic phosphate salts, 20-60 mg/kg/day, 4 to 6 intakes/daily	

N.B.: A progressive increase in the dose of phosphate supplements and active vitamin D analogs is recommended. The treatment should be personalized and adapted to the severity of the disease and patient's tolerance. The two medications must be administered in combination and at balanced dosages and monitored carefully.
Adapted and modified from Carpenter TO, et al. (2), Baroncelli GI, et al. (53), Linglart A, et al. (100), Haffner D, et al. (101), Rothenbuhler A, et al. (102), Trombetti A, et al. (103), and authors' expertise.

11.1.1 Burosumab treatment in patients with XLH

Burosumab, a recombinant human IgG1 monoclonal antibody that targets FGF23, has been approved by the US Food and Drug Administration (FDA), the European Medicines Agency (EMA), and the Japan's Health Authority (PMDA) for the treatment of patients with XLH. In Europe, burosumab is indicated in patients in children 1 year of age and older and adolescents with growing skeletons with radiographic evidence of rickets. The criteria for receiving burosumab vary between European countries. They are based according to eligibility rules for early access programs or qualification for reimbursement from health insurance or public health systems (123).

Some studies showed that burosumab treatment improved serum phosphate concentration and TmP/GFR values and were associated with rapid healing of radiographic sings of rickets, improved osteoarticular and muscular pain, and physical function (124, 125). Furthermore, a greater clinical improvement in rickets severity, longitudinal growth, and biochemical findings has been demonstrated in patients with XLH and an RSS of at least 2 treated with burosumab compared with patients continuing on conventional treatment (126). Beneficial effects of burosumab treatment on phosphate metabolism, skeletal lesions of rickets, and linear growth are reported in other recent studies (127–133). The main recommendations for treatment with burosumab for patients with XLH are summarized in Table 9 (134–136). Serum phosphate concentration represents a main biochemical parameter to titrate the dose of burosumab. Dose adjustments should be continued until the target low-normal serum phosphate concentration is reached or no further increase is observed after dose escalation (101, 135). Likely, targeting fasting serum phosphate at the lower end of the normal reference range for age is the safest approach to reduce the risk of ectopic mineralization (101). Serial measurement of alkaline phosphatase activity may also have a pivotal role to assess the progression of disease (135). A progressive decline in serum alkaline phosphatase activity, despite still being above the age- and sex-specific upper limit of normal, may indicate a sufficient clinical response (135). Serum phosphate concentrations below the age- and sex-specific low limit of normal may be considered as a sufficient treatment response if is associated with a sustained decrease in serum alkaline phosphatase activity and improved signs of rickets (135). Periodic measurements of serum 25(OH)D concentration are suggested, because a decline in vitamin D status may be the consequence of reduced sunlight exposure but also to the effect of burosumab treatment (14, 134, 135). We

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TABLE 8 Some general recommendations for the follow-up of patients with XLH receiving conventional treatment.

Clinical assessment	Timing
Clinical and auxological examination[a]	< 5 years, 1-3 months; > 5 years: 3-6 months
Odontostomatology examination	Every 6-12 months[b] or based on clinical symptoms
Orthopedic examination	Every 12 months[c] or based on clinical symptoms
Otolaryngology	> 8 years or based on clinical symptoms
Biochemical parameters	
Serum calcium, phosphate, creatinine, alkaline phosphatase, PTH	Every 3-6 months
Urinary calcium, phosphate, creatinine[d]	Every 3-6 months
Imaging examinations	
Radiographs of wrists[e], knees[e], standing lower limbs[f]	Every 1-2 years or based on clinical signs
Renal ultrasonography	Every year[g]
Brain magnetic resonance imaging	In the presence of craniosynostosis or skull shape malformation, headache, neurological symptoms or visual disturbances
Quality of life	Every year[h]

[a]Including measurement of height, weight, body mass index, pubertal stage, intercondylar and intermalleolar distance, head circumference with skull shape; presence of signs of rickets, pain, stiffness, fatigue; neurological evaluation (in patients with craniosynostosis or spinal stenosis).
[b]After tooth eruption.
[c]After initiation of walking.
[d]To assess urinary calcium excretion as calcium/creatinine ratio or calcium excretion 24h, and TmP/GFR.
[e]To assess the rickets severity score when appropriate.
[f]To assess and quantify the degree of varism or valgism.
[g]Every 6 months if nephrocalcinosis is diagnosed.
[h]Assessed by using age-appropriate and disease-appropriate scales for children and adolescents.
Adapted and modified from Carpenter TO, et al (2),, Baroncelli GI, et al. (53), Linglart A, et al. (100), Haffner D, et al. (101), Rothenbuhler A, et al. (102), Trombetti A, et al. (103), Rainmann A, et al. (122), and authors' expertise.

TABLE 9 Main recommendations for the treatment with burosumab in patients with XLH.

• If guidelines and reimbursement criteria allow, burosumab treatment should be started as early as possible in patients over the age of 1 year (6 months in some countries, such as the USA), mainly in those with severe rickets (RSS ≥ 2). If conventional therapy is used as initial treatment, a prompt switch to burosumab is required in cases of insufficient response.
• Conventional treatment must be discontinued at least 7-10 days before starting burosumab treatment.
• A starting dose of burosumab of 0.8 mg/kg body weight, given subcutaneously every 2 weeks is recommended.
• Titrate burosumab in 0.4 mg/kg increments to increase fasting serum phosphate concentration within the lower end of the normal reference range for age to a maximum dosage of 2.0 mg/kg body weight (maximum dose 90 mg).
• Assess fasting serum phosphate concentration during the titration period between injections, ideally 7-11 days after the last injection, to detect the effect of treatment and to avoid hyperphosphataemia.
• After achieving a steady state of serum phosphate concentration, which can be assumed after 3-4 moonths on a stable dosage, fasting serum phosphate should be measured preferentially directly before injections to detect underdosing.
• Burosumab treatment should be discontinued if fasting serum phosphate concentrations are above the upper range of normal for age[a].
• Serum phosphate concentration below the age- and sex-specific low-limit of normal may be acceptable if there is a sustained decrease in alkaline phosphatase activity, improvement of rickets, and the patient is responding clinically.
• Serum 25(OH)D concentration should be maintained at >50 nmol/L (20 ng/mL) to prevent secondary hyperparathyroidism and associated phosphaturia.
• Patients who have started treatment with burosumab should continue treatment throughout adolescence until the closure of the growth plate.
• Physiologic age-based parameters, such as growth plate closure, are likely the more appropriate indicators to determine when to change the burosumab dosing regimen from the pediatric (Q2W) to the adult dose (Q4W).
• A multidisciplinary evaluation should be conducted with the adult team during the transition phase to consider the followup of burosumab through adulthood.
• Changes in physical ability and quality of life in older patients may require the use of monitoring tools similar to those used in the adult population with XLH[b].

[a]According to Summary of Product Characteristics (https://www.ema.europa.eu/en/documents/product-information/crysvita-epar-product-information_en.pdf) the next dose should be withheld and the fasting serum phosphate concentration reassessed in 2 weeks. The patient must have serum phosphate below the reference range before restarting burosumab. Once serum phosphate is below the reference range, treatment may be restarted at approximately half the previous dose, administered every 4 weeks. Serum phosphate concentration should be reassessed 2 weeks after any change in dose.
[b]e.g. 6-minute walk test, handgrip strength, Brief Pain and Fatigue Inventories and the Western Ontario and McMaster Universities Arthritis Index (WOMAC).
From Haffner D, et al. (101), Trombetti A, et al. (103), Seefried L, et al. (123), Padidela R, et al. (134), and Mughal MZ, et al. (135), and authors' expertise.

recommend maintaining serum 25(OH)D >50 nmol/L (20 ng/mL) to prevent secondary hyperparathyroidism and to facilitate burosumab-mediated 1,25$(OH)_2$D synthesis (135). Measurement of serum 1,25$(OH)_2$D concentration during burosumab treatment is debated (14). A Consensus Statement recommended monitoring 1,25$(OH)_2$D during burosumab treatment to assess the increment of this vitamin D metabolite (101), but its role in adjusting burosumab dose has not been documented. In the opinion of other authors, measurement of serum 1,25$(OH)_2$D concentrations during burosumab adds cost without clear benefit, so it is not recommended (14).

Some recommendations for the follow-up of patients with XLH treated with burosumab are reported in Table 10. The timing of clinical evaluation and biochemical measurements may vary according to the expertise of the authors and should be tailored for each patient. Burosumab treatment is well tolerated and safe. The most common adverse drug reactions reported in pediatric patients were transient injection site reaction, headache, and pain in the extremities (101, 124, 132, 134–137).

11.1.2 Growth hormone treatment in patients with XLH

Some studies showed that growth hormone (GH) in association with conventional treatment may increase short-term linear growth in patients with XLH (53). However, the effects on final height are not conclusive (138–140). A recent large study suggested that continuing GH treatment in patients with XLH after switching from conventional therapy to burosumab, if the height prognosis

was compromised, might be beneficial for the final height (141). Nevertheless, GH treatment in association with conventional treatment (101) or burosumab is not recommended routinely, and further longer studies are required to examine the role of GH in addition to burosumab.

11.2 Other forms of FGF23-dependent hypophosphatemic bone disorders

Hypophosphatemic rickets may be a rare complication in some patients with non-lethal Raine syndrome (ARHR3) (78, 142–144) and in patients with fibrous dysplasia (145, 146). Hypophosphatemia has been reported in approximately 50% of patients with fibrous dysplasia/McCune Albright syndrome and is associated with rickets or osteomalacia. Serum FGF23 concentrations are increased due to a mass of FGF23-producing cells in fibrous bone lesions (145). Hypophosphatemic rickets may be treated with the conventional treatment by oral inorganic phosphate salts associated with vitamin D active metabolites.

TIO may be solved definitively by removing the tumor causing excessive production of FGF23 (23, 78, 147). Measurement of circulating FGF23 concentration may be useful to detect the progressive reduction after tumor resection or tumor recurrence (78). When tumor resection is not possible or the tumor is not found, and in patients awaiting surgery, conventional treatment similar to that used in patients with XLH should be administered (23, 24, 147).

11.2.1 Burosumab treatment in patients with other FGF23-dependent hypophosphatemic bone disorders

Burosumab has been administered in patients with other forms of rickets, including two adult patients with ARHR1 who showed normalization of serum phosphate concentrations, healing of pseudofracture, reduced fatigue and bone pain (148), and in two children with fibrous dysplasia (149, 150) whose serum phosphate concentrations normalized (149) and alkaline phosphatase activity progressively reduced (150). Furthermore, some case reports in pediatric patients with cutaneous skeletal hypophosphatemia syndrome (27–30) showed that burosumab improved phosphate metabolism, bone turnover, skeletal abnormalities, and quality of life, suggesting that it could be a promising treatment for patients with the FGF23-mediated hypophosphatemia associated with this rare disorder.

11.3 FGF23-independent hypophosphatemic rickets

Monotherapy with oral inorganic phosphate supplements is the usual treatment for patients with HHRH, whereas active vitamin D metabolites are contraindicated due to the increased $1,25(OH)_2D$ concentration (2, 151, 152). Treatment with phosphate should be targeted to decrease the $1,25(OH)_2D$ concentration, reduce intestinal calcium absorption and hypercalciuria, and improve bone mineralization (2). Potassium citrate, salt restriction, and hyperhydration may be useful tools to reduce hypercalciuria. Moreover, treatment with fluconazole (100 mg once daily) has been proposed to inhibit the 1α-hydroxylase and reduce $1,25(OH)_2D$ concentration and hypercalciuria to almost normal values (153). Furthermore, GH treatment associated with oral phosphate supplements improved the renal phosphate leakage and resulted in accelerated linear growth (154). The addition of GH, fluconazole,

TABLE 10 Some general recommendations for the follow-up of patients with XLH receiving burosumab treatment.

Clinical assessment	Timing
Clinical and auxological examination[a]	< 5 years: 1-3 months; > 5 years: 3-6 months
Odontostomatology examination[b]	Every 6-12 months or based on clinical symptoms
Orthopedic examination[c]	Every 12 months or based on clinical symptoms
Otolaryngology examination	> 8 years or based on clinical symptoms
Biochemical parameters	
Fasting serum calcium, phosphate, creatinine, alkaline phosphatase, PTH	Every 4 weeks during the first 3 months; thereafter at least every 3 months or based on clinical and biochemical pattern
Serum 25(OH)D and $1,25(OH)_2D$[d]	Every 3-4 months, mainly during the winter months
Urinary calcium, phosphate, creatinine[e]	Every 4 weeks during the first 3 months; thereafter at least every 3 months or based on clinical and biochemical pattern
Imaging examinations	
Radiographs of wrists[f], knees[f], standing lower limbs[g]	Every 1-2 years or based on clinical signs
Renal ultrasonography	Every 1-2 years[h]
Brain magnetic resonance imaging	In the presence of craniosynostosis or skull shape malformation, headache, neurological symptoms or visual disturbances
Quality of life monitoring	**Every 3-6 months up to 1-2 years[i]**
Other	
Monitoring adverse events[j]	Continuous active collection and reporting

[a]Including measurement of height, weight, body mass index, pubertal stage, intercondylar and intermalleolar distance, head circumference with skull shape; presence of signs of rickets, pain, stiffness, fatigue; neurologic evaluation (in patients with craniosynostosis or spinal stenosis).
[b]After tooth eruption.
[c]After initiation of walking.
[d]Adequate body stores of 25(OH)D may also facilitate burosumab-mediated $1,25(OH)_2D$ synthesis.
Measurement of serum $1,25(OH)_2D$ concentrations is not recommended by some authors (14).
[e]To assess urinary calcium excretion as calcium/creatinine ratio or calcium excretion 24h, and TmP/GFR.
[f]To assess the rickets severity score, when appropriate.
[g]To assess and quantify the degree of varism or valgism.
[h]Every year if nephrocalcinosis is diagnosed in patients who had received conventional treatment.
[i]Assessed by using age-appropriate and disease-appropriate scales for children and adolescents. The timing of evaluation may vary according to the clinical symptoms.
[j]In many European Countries healthcare professionals are asked to report any suspected adverse reactions to specific national databases.
Adapted and modified from Haffner D, et al. (101), Trombetti A, et al. (103), Rainmann A, et al. (122), Padidela R, et al. (134), Mughal MZ, et al. (135), and authors' expertise.

and salt restriction improved the effect of the conventional therapy with phosphate supplementation and potassium citrate (155). However, additional studies are required to provide the long-term efficacy and safety of fluconazole and GH alone or in combination.

Phosphate supplements and vitamin D active metabolites are suggested for the treatment of patients with hypophosphatemic rickets secondary to tubulopathies, Fanconi syndrome, Dent disease, or other systemic diseases, in association with the recommended treatments for each disease (2).

12 Conclusions

Rickets should be considered a health priority in infants and children. A correct clinical and biochemical approach is fundamental to identify the subjects with suspected rickets. Early diagnosis and adequate treatment are primary targets to avoid severe consequences later in life. The differentiation between the nutritional forms from the genetic forms of rickets is an important step for the correct treatment. Each form of rickets requires a specific diagnostic trail, distinct treatment and an interdisciplinary approach to management (53, 103, 123). Some algorithms to facilitate the diagnostic approach are suggested. Furthermore, some recommendations for the most appropriate treatments for patients with rare forms of hereditary rickets are proposed.

Author contributions

GB: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. PC: Conceptualization, Data curation, Formal analysis, Investigation, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. TA: Writing – original draft, Writing – review & editing. FB: Writing – original draft, Writing – review & editing. AC: Writing – original draft, Writing – review & editing. MC: Writing – original draft, Writing – review & editing. MC: Writing – original draft, Writing – review & editing. LD: Writing – original draft, Writing – review & editing. ND: Writing – original draft, Writing – review & editing. MF: Writing – original draft, Writing – review & editing. DF: Writing – original draft, Writing – review & editing. RF: Writing – original draft, Writing – review & editing. MK: Writing – original draft, Writing – review & editing. SL: Writing – original draft, Writing – review & editing. MM: Writing – original draft, Writing – review & editing. MP: Writing – original draft, Writing – review & editing. AS: Writing – original draft, Writing – review & editing. DT: Writing – original draft, Writing – review & editing. FV: Writing – original draft, Writing – review & editing. MW: Writing – original draft, Writing – review & editing. GW: Writing – original draft, Writing – review & editing. SM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Munns CF, Shaw N, Kiely M, Specker BL, Thacher TD, Ozono K, et al. Global consensus recommendations on prevention and management of nutritional rickets. *J Clin Endocrinol Metab*. (2016) 101:394–415. doi: 10.1210/jc.2015-2175

2. Carpenter TO, Shaw NJ, Portale AA, Ward LM, Abrams SA, Pettifor JM. Rickets. *Nat Rev Dis Primers*. (2017) 3:17101. doi: 10.1038/nrdp.2017.101

3. Thacher TD. Calcium-deficiency rickets. In: Hochberg Z, editor. *Vitamin D and rickets*. Karger AG, P.O. Box, CH-4009 Basel, Switzerland (2003). p. 105–25.

4. Haffner D, Leifheit-Nestler M, Grund A, Schnabel D. Rickets guidance: part I-diagnostic workup. *Pediatr Nephrol*. (2022) 37:2013–36. doi: 10.1007/s00467-021-05328-w

5. Chacham S, Pasi R, Chegondi M, Ahmad N, Mohanty SB. Metabolic bone disease in premature neonates: An unmet challenge. *J Clin Res Pediatr Endocrinol*. (20202020) 12:332–9. doi: 10.4274/jcrpe.galenos.2019.2019.0091

6. Imel EA, Econs MJ. Fibroblast growth factor 23: roles in health and disease. *J Am Soc Nephrol*. (2005) 16:2565–75. doi: 10.1681/ASN.2005050573

7. Beck-Nielsen SS, Brock-Jacobsen B, Gram J, Brixen K, Jensen TK. Incidence and prevalence of nutritional and hereditary rickets in Southern Denmark. *Eur J Endocrinol*. (2009) 160:491–7. doi: 10.1530/EJE-08-0818

8. Rafaelsen S, Johansson S, Raeder H, Bjerknes R. Hereditary hypophosphatemia in Norway: a retrospective population-based study of genotypes, phenotypes, and treatment complications. *Eur J Endocrinol*. (2016) 174:125–36. doi: 10.1530/EJE-15-0515

9. Beck-Nielsen SS, Mughal Z, Haffner D, Nilsson O, Levtchenko E, Ariceta G, et al. FGF23 and its role in X-linked hypophosphatemia-related morbidity. *Orphanet J Rare Dis*. (2019) 14:58. doi: 10.1186/s13023-019-1014-8

10. Pavone V, Testa G, Gioitta Iachino S, Evola FR, Avondo S, Sessa G. Hypophosphatemic rickets: etiology, clinical features and treatment. *Eur J Orthop Surg Traumatol*. (2015) 25:221–6. doi: 10.1007/s00590-014-1496-y

11. Eltan M, Alavanda C, Yavas Abali Z, Ergenekon P, Yalındag Ozturk N, Saka M, et al. A rare cause of hypophosphatemia: raine syndrome changing clinical features with age. *Calcif Tissue Int*. (2020) 107:96–103. doi: 10.1007/s00223-020-00694-3

12. Boyce AM, Collins MT. Fibrous dysplasia/McCune-Albright syndrome: a rare, mosaic disease of Gα_s activation. *End Rev*. (2020) 41:345–70. doi: 10.1210/endrev/bnz011

13. Brownstein CA, Adler F, Nelson-Williams C, Iijima J, Li P, Imura A, et al. A translocation causing increased α-Klotho level results in hypophosphatemic rickets and hyperparathyroidism. *Proc Natl Acad Sci USA*. (2008) 105:3455–60. doi: 10.1073/pnas.0712361105

14. Ackah SA, Imel EA. Approach to hypophosphatemic rickets. *J Clin Endocrinol Metab*. (2023) 108:209–20. doi: 10.1210/clinem/dgac488

15. Cormier-Daire V, Delezoide AL, Philip N, Marcorelles P, Casas K, Hillion Y, et al. Clinical, radiological, and chondro-osseous findings in opsismodysplasia: survey of a series of 12 unreported cases. *J Med Genet*. (2003) 40:195–200. doi: 10.1136/jmg.40.3.195

16. Brown WW, Juppner H, Langman CB, Price H, Farrow EG, White KE, et al. Hypophosphatemia with elevations in serum fibroblast growth factor 23 in a child with Jansen's metaphyseal chondrodysplasia. *J Clin Endocrinol Metab*. (2009) 94:17–20. doi: 10.1210/jc.2008-0220

17. Fukumoto S. Targeting fibroblast growth factor 23 signaling with antibodies and inhibitors, is there a rationale? *Front Endocrinol*. (2018) 9:48. doi: 10.3389/fendo.2018.00048

18. Shore RM. Disorders of phosphate homeostasis in children, part 2: hypophosphatemic and hyperphosphatemic disorders. *Pediatr Radiol*. (2022) 52:2290–305. doi: 10.1007/s00247-022-05373-z

19. Amatschek S, Haller M, Oberbauer R. Renal phosphate handling in human: what can we learn from hereditary hypophosphataemias? *Eur J Clin Invest*. (2010) 40:552–60. doi: 10.1111/j.1365-2362.2010.02286.x

20. Carpenter TO. The expanding family of hypophosphatemic syndromes. *J Bone Miner Metab*. (2012) 30:1–9. doi: 10.1007/s00774-011-0340-2

21. Levine MA. Diagnosis and management of vitamin D dependent rickets. *Front Pediatr*. (2020) 8:315. doi: 10.3389/fped.2020.00315

22. Roizen JD, Li D, O'Lear L, Javaid MK, Shaw NJ, Ebeling PR, et al. CYP3A4 mutation causes vitamin D-dependent rickets type 3. *J Clin Invest*. (2018) 128:1913–18. doi: 10.1172/JCI98680

23. Florenzano P, Hartley IR, Jimenez M, Roszko K, Gafni RI, Collins MT. Tumor-induced osteomalacia. *Calcif Tissue Int*. (2021) 108:128–42. doi: 10.1007/s00223-020-00691-6

24. Bosman A, Palermo A, Vanderhulst J, De Beur SMJ, Fukumoto S, Minisola S, et al. Tumor-induced osteomalacia: a systematic clinical review of 895 cases. *Calcif Tissue Int*. (2022) 111:367–79. doi: 10.1007/s00223-022-01005-8

25. Haeusler G, Freilinger M, Dominkus M, Egerbacher M, Amann G, Kolb A, et al. Tumor- tumor-induced hypophosphatemic rickets in an adolescent boy: clinical presentation, diagnosis, and histological findings in growth plate and muscle tissue. *J Clin Endocrinol Metab*. (2010) 95:4511–7. doi: 10.1210/jc.2010-0543

26. Crossen SS, Zambrano E, Newman B, Bernstein JA, Messner AH, Bachrach L, et al. Tumor-induced osteomalacia in a 3-year-old with unresectable central giant cell lesions. *J Pediatr Hematol Oncol*. (2017) 39:e21–4. doi: 10.1097/MPH.0000000000000686

27. Khadora M, Mughal MZ. Burosumab treatment in a child with cutaneous skeletal hypophosphatemia syndrome: A case report. *Bone Rep*. (2021) 15:101138. doi: 10.1016/j.bonr.2021.101138

28. Huynh C, Gillis A, Fazendin J, Abdullatif H. A case report to assess the safety and efficacy of burosumab, an investigational antibody to FGF23, in a single pediatric patient with epidermal nevus syndrome and associated hypophosphatemic rickets. *Bone Rep*. (2022) 17:101605. doi: 10.1016/j.bonr.2022.101605

29. Merz LM, Buerger F, Ziegelasch N, Zenker M, Wieland I, Tobias Lipek T, et al. A case report: first long-term treatment with burosumab in a patient with cutaneous-skeletal hypophosphatemia syndrome. *Front Endocrinol*. (2022) 13:866831. doi: 10.3389/fendo.2022.866831

30. Sugarman J, Maruri A, Hamilton DJ, Tabatabai L, Luca D, Cimms T, et al. The efficacy and safety of burosumab in two patients with cutaneous skeletal hypophosphatemia syndrome. *Bone*. (2023) 166:116598. doi: 10.1016/j.bone.2022.116598

31. Wolf M, Rubin J, Achebe M, Econs MJ, Peacock M, Imel EA, et al. Effects of iron isomaltoside vs ferric carboxymaltose on hypophosphatemia in iron-deficiency anemia: two randomized clinical trials. *JAMA*. (2020) 323:432–43. doi: 10.1001/jama.2019.22450

32. Hall AM, Bass P, Unwin RJ. Drug-induced renal Fanconi syndrome. *QJM*. (20142014) 107:261–9. doi: 10.1093/qjmed/hct258

33. Foreman JW. Fanconi syndrome. *Pediatr Clin North Am*. (20192019) 66:159–67. doi: 10.1016/j.pcl.2018.09.002

34. Bikle DD. Vitamin D insufficiency/deficiency in gastrointestinal disorders. *J Bone Miner Res*. (2007) 22:V50–4. doi: 10.1359/JBMR.07S208

35. Lambert AS, Linglart A. Hypocalcemic and hypophosphatemic rickets. *Best Pract Res Clin Endocrinol Metab*. (2018) 32:455–76. doi: 10.1016/j.beem.2018.05.009

36. Otero JE, Gottesman GS, McAlister WH, Mumm S, Madson KL, Kiffer-Moreira T, et al. Severe skeletal toxicity from protracted etidronatetherapy for generalized arterial calcification of infancy. *J Bone Miner Res*. (2013) 28:419–30. doi: 10.1002/jbmr.1752

37. Miyai K, Ariyasu D, Numakura C, Yoneda K, Nakazato H, Hasegawa Y. Hypophosphatemic rickets developed after treatment with etidronate disodium in a patient with generalized arterial calcification in infancy. *Bone Rep*. (2015) 3:57–60. doi: 10.1016/j.bonr.2015.09.001

38. Linglart A, Biosse-Duplan M. Hypophosphatasia. *Curr Osteoporos Rep*. (2016) 3:95–105. doi: 10.1007/s11914-016-0309-0

39. Whyte MP, Wenkert D, Zhang F. Hypophosphatasia: natural history study of 101 affected children investigated at one research center. *Bone*. (2016) 93:125–38. doi: 10.1016/j.bone.2016.08.019

40. Foldes J, Balena R, Ho A, Parfitt AM, Kleerekoper M. Hypophosphatemic rickets with hypocalciuria following long-term treatment with aluminum-containing antacid. *Bone*. (1991) 12:67–71. doi: 10.1016/8756-3282(91)90002-Z

41. Vukicevic S. First case of rickets following prolonged treatment with aluminum containing antacids that bind phosphate. *Bone*. (1992) 13:119. doi: 10.1016/8756-3282(92)90449-7

42. Teotia M, Teotia SP, Singh KP. Endemic chronic fluoride toxicity and dietary calcium deficiency interaction syndromes of metabolic bone disease and deformities in India: year 2000. *Indian J Pediatr*. (1998) 65:371–81. doi: 10.1007/BF02761130

43. Pettifor JM. Screening for nutritional rickets in a community. *J Steroid Biochem Mol Biol*. (2016) 164:139–44. doi: 10.1016/j.jsbmb.2015.09.008

44. Heimburger DC. Clinical manifestations of nutrient deficiencies and toxicities. In: Ross AC, Cabellero B, Cousins RJ, Tucker KL, Ziegler TR, editors. *Modern nutrition in health and disease*, *11th ed*. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia (2014). p. 757–70.

45. Holick MF. Resurrection of vitamin D deficiency and rickets. *J Clin Invest*. (2006) 116:2062–72. doi: 10.1172/JCI29449

46. Lerch C, Meissner T. Interventions for the prevention of nutritional rickets in term born children. *Cochrane Database Syst Rev*. (2007) 4:CD006164. doi: 10.1002/14651858.CD006164.pub2

47. Wagner CL, Greer FR. American Academy of Pediatrics Section on Breastfeeding, American Academy of Pediatrics Committee on Nutrition. Prevention of rickets and vitamin D deficiency in infants, children, and adolescents. *Pediatrics*. (2008) 122:1142–52. doi: 10.1542/peds.2008-1862

48. Misra M, Pacaud D, Petryk A, Collett-Solberg PF, Kappy M. Drug and Therapeutics Committee of the Lawson Wilkins Pediatric Endocrine Society. Vitamin D deficiency in children and its management: review of current knowledge and recommendations. *Pediatrics*. (2008) 122:398–417. doi: 10.1542/peds.2007-1894

49. Baroncelli GI, Carlucci G, Freri E, Giuca MR, Guarnieri V, Navarra G, et al. The diagnosis of hypophosphatasia in children as a multidisciplinary effort: an expert opinion. *J Endocrinol Invest*. (2024) 47:739–47. doi: 10.1007/s40618-023-02199-w

50. Baroncelli GI, Sessa MR, Pelosini C, Bertelloni S, Michelucci A, Toschi B, et al. Intact FGF23 concentration in healthy infants, children, and adolescents, and diagnostic usefulness in patients with X-linked hypophosphatemic rickets. *J Endocrinol Invest*. (2024) 47:873–82. doi: 10.1007/s40618-023-02202-4. in press.

51. Tiosano D, Hochberg Z. Hypophosphatemia: the common denominator of all rickets. *J Bone Miner Metab*. (2009) 27:392–401. doi: 10.1007/s00774-009-0079-1

52. Stark H, Eisenstein B, Tieder M, Rachmel A, Alpert G. Direct measurement of TP/GFR: a simple and reliable parameter of renal phosphate handling. *Nephron*. (1986) 44:125–8. doi: 10.1159/000184216

53. Baroncelli GI, Mora S. (2021) X-linked hypophosphatemic rickets: multisystemic disorder in children requiring multidisciplinary management *Front Endocrinol*. 12:688309. doi: 10.3389/fendo.2021.688309

54. Thacher TD, Fischer PR, Pettifor JM, Lawson JO, Isichei CO, Reading JC, et al. A comparison of calcium, vitamin D, or both for nutritional rickets in Nigerian children. *N Engl J Med*. (1999) 341:563–8. doi: 10.1056/NEJM199908193410803

55. Baroncelli GI, Bereket A, El Kholy M, Audì L, Cesur Y, Ozkan B, et al. Rickets in the Middle East: role of environment and genetic predisposition. *J Clin Endocrinol Metab*. (2008) 93:1743–50. doi: 10.1210/jc.2007-1413

56. Dahifar H, Faraji A, Ghorbani A, Yassobi S. Impact of dietary and lifestyle on vitamin D in healthy student girls aged 11–15 years. *J Med Investig*. (2006) 53:204–8. doi: 10.2152/jmi.53.204

57. Agarwal A, Gulati D. Early adolescent nutritional rickets. *J Orthop Surg*. (2009) 17:340–5. doi: 10.1177/230949900901700320

58. Hazzazi M, Alzeer I, Tamimi W, Al Atawi M, Al Alwan I. Clinical presentation and etiology of osteomalacia/rickets in adolescents. *Saudi J Kidney Dis Transplant*. (2013) 24:938–4. doi: 10.4103/1319-2442.118087

59. Atapattu N, Shaw N, Hogler W. Relationship between serum 25-hydroxyvitamin D and parathyroid hormone in the search for a biochemical definition of vitamin D deficiency in children. *Pediatr Res*. (2013) 74:552–6. doi: 10.1038/pr.2013.139

60. Rios-Leyvraz M, Thacher TD, Dabas A, Hassan Elsedfy H, Baroncelli GI, Cashman KD. Serum 25-hydroxyvitamin D threshold and risk of rickets in young children: a systematic review and individual participant data meta-analysis to inform the development of dietary requirements for vitamin D. *Eur J Nutr*. (2024) 63:673–95. doi: 10.1007/s00394-023-03299-2

61. El Kholy M, Elsedfy H, Fernandez-Cancio M, Hamza RT, Amr NH, Ahmed AY, et al. Nutritional rickets: vitamin D, calcium, and the genetic make-up. *Pediatr Res*. (2017) 81:356–63. doi: 10.1038/pr.2016.222

62. Molin A, Wiedemann A, Demers N, Kaufmann M, Do Cao J, Mainard L, et al. Vitamin D-dependent rickets type 1B (25-hydroxylase deficiency): a rare condition or a misdiagnosed condition? *J Bone Miner Res*. (2017) 32:1893–9. doi: 10.1002/jbmr.3181

63. Kubota T, Kitaokaa T, Miura K, Fujiwara M, Ohata Y, Miyoshi Y, et al. Serum fibroblast growth factor 23 is a useful marker to distinguish vitamin D-deficient rickets from hypophosphatemic rickets. *Horm Res Paediatr*. (2014) 81:251–7. doi: 10.1159/000357142

64. Prentice A, Ceesay M, Nigdikar S, Allen SJ, Pettifor JM. FGF23 is elevatewd in Gambian children with rickets. *Bone*. (2008) 42:788–97. doi: 10.1016/j.bone.2007.11.014

65. States LJ. Imaging of rachitic bone. In: Hochberg Z, editor. *Vitamin D and rickets*, vol. . p . Karger AG, P.O. Box, CH-4009 Basel, Switzerland (2003). p. 80–92.

66. Warthon B, Bishop N. Rickets. *Lancet*. (2003) 362:1389–400. doi: 10.1016/S0140-6736(03)14636-3

67. Shaw NJ. Vitamin D deficiency rickets. In: Hochberg Z, editor. *Vitamin D and rickets*, vol. . p . Karger AG, P.O. Box, CH-4009 Basel, Switzerland (2003). p. 93–104.

68. Chapman T, Sugar N, Done S, Marasigan J, Wambold N, Feldman K. Fractures in infants and toddlers with rickets. *Pediatr Radiol*. (2010) 40:1184–9. doi: 10.1007/s00247-009-1470-8

69. Thacher TD, Fischer PR, Pettifor JM, Lawson JO, Manaster BJ, Reading JC. Radiographic scoring method for the assessment of the severity of nutritional rickets. *J Trop Pediatr*. (2000) 46:132–9. doi: 10.1093/tropej/46.3.132

70. Thacher TD, Pettifor JM, Tebben PJ, Creo AL, Skrinar A, Mao M, et al. Rickets severity predicts clinical outcomes in children with X-linked hypophosphatemia: utility of the radiographic rickets severity score. *Bone*. (2019) 122:76–81. doi: 10.1016/j.bone.2019.02.010

71. Whyte MO, Fujita KP, Moseley S, Thompson DD, McAlister WH. Validation of a novel scoring system for changes in skeletal manifestations of hypophosphatasia in newborns, infants, and children: the radiographic global impression of change scale. *J Bone Miner Res*. (2018) 33:868–74. doi: 10.1002/jbmr.3377

72. Lim R, Shailam R, Hulett R, Skrinar A, Nixon A, Williams A, et al. Validation of the radiographic global impression of change (RGI-C) score to assess healing of rickets in pediatric X-linked hypophosphatemia (XLH). *Bone*. (2021) 148:115964. doi: 10.1016/j.bone.2021.115964

73. Capelli S, Donghi V, Maruca K, Vezzoli G, Corbetta S, Brandi ML, et al. Clinical and molecular heterogeneity in a large series of patients with hypophosphatemic rickets. *Bone*. (2015) 79:143–9. doi: 10.1016/j.bone.2015.05.040

74. Yasuhisa Ohata Y, Ishihara Y. Pathogenic variants of the PHEX gene. *Endocrines*. (2022) 3:498–511. doi: 10.3390/endocrines3030040

75. Baroncelli GI, Vierucci F, Bertelloni S. Rachitismo da deficit di vitamina D: indicazioni per la diagnosi e la terapia. In: Baroncelli GI, editor. *Ipovitaminosi D. Prevenzione e trattamento nel neonato, nel bambino e nell'adolescente*, vol. p . Edizioni Mattioli, Fidenza, Italy (2011). p. 99–121.

76. Cesur Y, Çaksen H, Gündem A, Kirimi E, Odabas D. Comparison of low and high dose of vitamin D treatment in nutritional vitamin D deficiency rickets. *J Pediatr Endocrinol Metab*. (2003) 16:1105–9. doi: 10.1515/jpem.2003.16.8.1105

77. Mittal H, Rai S, Shah D, Madhu SV, Mehrotra G, Malhotra RK, et al. 300,000 IU or 600,000 IU of oral vitamin D3 for treatment of nutritional rickets: a randomized controlled trial. *Indian Pediatr*. (2014) 51:265–72. doi: 10.1007/s13312-014-0399-7

78. Haffner D, Leifheit-Nestler M, Grund A, Schnabel D. Rickets guidance: part II-management. *Pediatr Nephrol*. (2022) 37:2289–302. doi: 10.1007/s00467-022-05505-5

79. Pearce SH, Cheetham TD. Diagnosis and management of vitamin D deficiency. *BMJ*. (2010) 340:b5664. doi: 10.1136/bmj.b5664

80. Tripkovic L, Lambert H, Hart K, Smith CP, Bucca G, Penson S, et al. Comparison of vitamin D_2 and vitamin D_3 supplementation in raising serum 25-hydroxyvitamin D status: a systematic review and meta-analysis. *Am J Clin Nutr*. (2012) 95:1357–64. doi: 10.3945/ajcn.111.031070

81. Allgrove J, Shaw NJ. A practical approach to vitamin D deficiency and rickets. In: Allgrove J, Shaw NJ, editors. *Calcium and bone disorders in children and adolescents*. Karger AG, P.O. Box, CH-4009 Basel, Switzerland (2015). p. 119–33.

82. Baroncelli GI, Bertelloni S, Ceccarelli C, Amato V, Saggese G. Bone turnover in children with vitamin D deficiency rickets before and during treatment. *Acta Paediatr*. (2000) 89:513–8. doi: 10.1080/080352500750027763

83. Thacher TD, Smith L, Fischer PR, Isichei CO, Cha SS, Pettifor JM. Optimal dose of calcium for treatment of nutritional rickets: a randomized controlled trial. *J Bone Miner Res*. (2016) 31:2024–31. doi: 10.1002/jbmr.2886

84. Thacher TD, Fischer PR, Pettifor JM. Vitamin D treatment in calcium-deficiency rickets: a randomised controlled trial. *Arch Dis Child*. (2014) 99:807–11. doi: 10.1136/archdischild-2013-305275

85. Faienza MF, D'Amato E, Natale MP, Grano M, Chiarito M, Brunetti G, et al. Metabolic bone disease of prematurity: diagnosis and management. *Front Pediatr*. (2019) 7:143. doi: 10.3389/fped.2019.00143

86. Chinoy A, Mughal MZ, Raja Padidela R. Metabolic bone disease of prematurity: causes, recognition, prevention, treatment and longterm consequences. *Arch Dis Child Fetal Neonatal Ed*. (2019) 104:F560–6. doi: 10.1136/archdischild-2018-316330

87. Abrams SACommittee on Nutrition. Calcium and vitamin D requirements of enterally fed preterm infants. *Pediatrics*. (2013) 131:e1676–83. doi: 10.1542/peds.2013-0420

88. Agostoni C, Buonocore G, Carnielli VP, De Curtis M, Darmaun D, Decsi T, et al. Enteral nutrient supply for preterm infants: commentary from the European Society of Paediatric Gastroenterology, Hepatology and Nutrition Committee on Nutrition. *J Pediatr Gastroenterol Nutr*. (2010) 50:85–91. doi: 10.1097/MPG.0b013e3181adaee0

89. Koletzko B, Poindexter B, Uauy R. *Nutritional care of preterm infants: scientific basis and practical guidelines*. Switzerland: Karger AG P.O. Box, CH-4009 Basel (2014). doi: 10.1159/isbn.978-3-318-02641-2

90. Nehra D, Carlson SJ, Fallon EM, Kalish B, Potemkin AK, Gura KM, et al. A.S.P.E.N. clinical guidelines: nutrition support of neonatal patients at risk for metabolic bone disease. *J Parenter Enteral Nutr*. (2013) 37:570–98. doi: 10.1177/0148607113487216

91. Lee B, De Beritto T. Metabolic bone disease of prematurity. *Neoreviews*. (2022) 23:e311–8. doi: 10.1542/neo.23-5-e311

92. Abrams SA. Vitamin D in preterm and full-term infants. *Ann Nutr Metab*. (2020) 76:6–14. doi: 10.1159/000508421

93. Reade TM, Scriver CR, Glorieux FH, Nogrady B, Delvin E, Poirier R, et al. Response to crystalline 1alpha-hydroxyvitamin D_3 in vitamin D dependency. *Pediatr Res*. (1975) 9:593–9. doi: 10.1203/00006450-197507000-00008

94. Delvin EE, Glorieux FH, Marie PJ. Pettifor JM Vitamin D dependency: replacement therapy with calcitriol. *J Pediatr*. (1981) 99:26–34. doi: 10.1016/s0022-3476(81)80952-3

95. Miller WL, Portale AA. Vitamin D biosynthesis and vitamin D 1 α-hydroxylase deficiency. In: Hochberg Z, editor. *Vitamin D and rickets*. Karger AG, P.O. Box, CH-4009 Basel, Switzerland (2003). p. 156–74.

96. Thacher TD, Fischer PR, Singh RJ, Roizen J, Levine MA. CYP2R1 mutations impair generation of 25-hydroxyvitamin D and cause an atypical form of vitamin D deficiency. *J Clin Endocrinol Metab*. (2015) 100:E1005–13. doi: 10.1210/jc.2015-1746

97. Chaturvedi D, Garabedian M, Carel J-C, Léger J. Different mechanisms of intestinal calcium absorption at different life stages: therapeutic implications and long-term responses to treatment in patients with hereditary vitamin D-resistant rickets. *Horm Res Paediatr*. (2012) 78:326–31. doi: 10.1159/000341405

98. Marx SJ, Bliziotes MM, Nanes M. Analysis of the relation between alopecia and resistance to 1,25-dihydroxyvitamin D. *Clin Endocrinol (Oxf.)*. (1986) 25:373–81. doi: 10.1111/j.1365-2265.1986.tb01703.x

99. Malloy PJ, Feldman D. Hereditary 1,25-dihydroxyvitamin D-resistant rickets. In: Hochberg Z, editor. *Vitamin D and rickets*, vol. . p . Karger AG, P.O. Box, CH-4009 Basel, Switzerland (2003). p. 175–99.

100. Linglart A, Biosse-Duplan M, Briot K, Chaussain C, Esterle L, Guillaume-Czitrom S, et al. Therapeutic management of hypophosphatemic rickets from infancy to adulthood. *Endocr Connect*. (2014) 3:R13–30. doi: 10.1530/EC-13-0103

101. Haffner D, Emma F, Eastwood DM, Biosse Duplan M, Bachetta J, Schnabel D, et al. Clinical practice recommendations for the diagnosis and management of X-linked hypophosphataemia. *Nat Rev Nephrol*. (2019) 15:435–55. doi: 10.1038/s41581-019-0152-5

102. Rothenbuhler A, Schnabel D, Högler W, Linglart A. Diagnosis, treatment-monitoring and follow-up of children and adolescents with X-linked hypophosphatemia (XLH). *Metabolism*. (2020) 103S:153892. doi: 10.1016/j.metabol.2019.03.009

103. Trombetti A, Al-Daghri N, Brandi ML, Cannata-Andia JB, Cavalier E, Chandran M, et al. Interdisciplinary management of FGF23-related phosphate wasting syndromes: a Consensus Statement on the evaluation, diagnosis and care of patients with X-linked hypophosphataemia. *Nat Rev Endocrinol*. (2022) 18:366–84. doi: 10.1038/s41574-022-00662-x

104. Makitie O, Doria A, Kooh SW, Cole WG, Daneman A, Sochett E. Early treatment improves growth and biochemical and radiographic outcome in X-linked hypophosphatemic rickets. *J Clin Endocrinol Metab*. (2003) 88:3591–7. doi: 10.1210/jc.2003-030036

105. Zivičňjak M, Schnabel D, Staude H, Even G, Marx M, Beetz R, et al. Three-year growth hormone treatment in short children with X-linked hypophosphatemic rickets: effects on linear growth and body disproportion. *J Clin Endocrinol Metab*. (2011) 96:E2097–105. doi: 10.1210/jc.2011-0399

106. Fuente R, Gil-Peña H, Claramunt-Taberner D, Hernández O, Fernández-Iglesias A, Alonso-Durán L, et al. X-linked hypophosphatemia and growth. *Rev Endocr Metab Disord*. (2017) 18:107–15. doi: 10.1007/s11154-017-9408-1

107. Mao M, Carpenter TO, Whyte MP, Skrinar A, Chen CY, San Martin J, et al. Growth curves for children with X-linked hypophosphatemia. *J Clin Endocrinol Metab*. (2020) 105:1–7. doi: 10.1210/clinem/dgaa495

108. Chaussain-Miller C, Sinding C, Septier D, Wolikow M, Goldberg M, Garabedian M. Dentin structure in familial hypophosphatemic rickets: benefits of vitamin D and phosphate treatment. *Oral Dis*. (2007) 13:482–9. doi: 10.1016/j.metabol.2019.03.009

109. Connor J, Olear EA, Insogna KL, Katz L, Baker S, Kaur R, et al. Conventional therapy in adults with X-linked hypophosphatemia: effects on enthesopathy and dental disease. *J Clin Endocrinol Metab*. (2015) 100:3625–32. doi: 10.1210/JC.2015-2199

110. Baroncelli GI, Angiolini M, Ninni E, Galli V, Saggese R, Giuca MR. Prevalence and pathogenesis of dental and periodontal lesions in children with X-linked hypophosphatemic rickets. *Eur J Paediatr Dent*. (2006) 7:61–6.

111. Souza MA, Soares LA, Santos MA, Vaisbich MH. Dental abnormalities and oral health in patients with hypophosphatemic rickets. *Clinics*. (2010) 65:1023–6. doi: 10.1590/s1807-59322010001000017

112. Imel EA, Biggin A, Schindeler A, Munns CF. FGF23, hypophosphatemia, and emerging treatments. *J Bone Miner Res Plus*. (2019) 3:e10190. doi: 10.1002/jbm4.10190

113. Kapelari K, Köhle J, Kotzot D, Högler W. Iron supplementation associated with loss of phenotype in autosomal dominant hypophosphatemic rickets. *J Clin Endocrinol Metab*. (2015) 100:3388 –92. doi: 10.1210/jc.2015-2391

114. Hogler W, Kapelari K. Oral iron for prevention and treatment of rickets and osteomalacia in autosomal dominant hypophosphatemia. *J Bone Miner Res*. (2020) 35:226–30. doi: 10.1002/jbmr.3941

115. Saito T, Shimizu Y, Hori M, Taguchi M, Igarashi T, Fukumoto S, et al. A patient with hypophosphatemic rickets and ossification of posterior longitudinal ligament caused by a novel homozygous mutation in ENPP1 gene. *Bone*. (2011) 49:913–6. doi: 10.1016/j.bone.2011.06.029

116. Kotwal A, Ferrer A, Kumar R, Singh RJ, Murthy V, Schultz-Rogers L, et al. Clinical and biochemical phenotypes in a family with ENPP1 mutations. *J Bone Min Res*. (2020) 35:662–70. doi: 10.1002/jbmr.3938

117. Hoppner J, Kornak U, Sinningen K, Rutsch F, Oheim R, Grasemanna C. Autosomal recessive hypophosphatemic rickets type 2 (ARHR2) due to ENPP1-deficiency. *Bone*. (2021) 153:116111. doi: 10.1016/j.bone.2021.116111

118. Lorenz-Depiereux B, Schnabel D, Tiosano D, Hausler G, Strom TM. Loss-of-function ENPP1 mutations cause both generalized arterial calcification of infancy and autosomal-recessive hypophosphatemic rickets. *Am J Hum Genet*. (2010) 86:267–72. doi: 10.1016/j.ajhg.2010.01.006

119. Levy-Litan V, Hershkovitz E, Avizov L, Leventhal N, Bercovich D, Chalifa-Caspi V, et al. Autosomal-recessive hypophosphatemic rickets is associated with an inactivation mutation in the ENPP1 gene. *Am J Hum Genet*. (2010) 86:273–8. doi: 10.1016/j.ajhg.2010.01.010

120. Ferreira CR, Ziegler SG, Gupta C, Groden C, Hsu KS, Gahl WA. Treatment of hypophosphatemic rickets in generalized arterial calcification of infancy (GACI) without worsening of vascular calcification. *Am J Med Genet*. (2016) 170A:1308–11. doi: 10.1002/ajmg.a.37574

121. Ferreira CR, Hackbarth ME, Ziegler SG, Pan KS, Roberts MS, Rosing DR, et al. Prospective phenotyping of long-term survivors of generalized arterial calcification of infancy (GACI). *Genet Med*. (2021) 23:396–407. doi: 10.1038/s41436-020-00983-0

122. Raimann A, Mindler GT, Kocijan R, Bekes K, Zwerina J, Haeusler G, et al. Multidisciplinary patient care in X-linked hypophosphatemic rickets: one challenge, many perspectives. *Wien Med Wochenschr*. (2020) 170:116–23. doi: 10.1007/s10354-019-00732-2

123. Seefried L, Alzahrani A, Sancho PA, Bacchetta J, Crowley R, Emma F, et al. XLH Matters 2022: Insights and recommendations to improve outcomes for people living with X-linked hypophosphataemia (XLH). *Orphanet J Rare Dis*. (2023) 18:333. doi: 10.1186/s13023-023-02883-3

124. Carpenter TO, Whyte MP, Imel EA, Boot AM, Hogler W, Linglart A, et al. Burosumab therapy in children with X-linked hypophosphatemia. *N Engl J Med*. (2018) 378:1987–98. doi: 10.1056/NEJMoa1714641

125. Whyte MP, Carpenter TO, Gottesman GS, Mao M, Skrinar A, San Martin J, et al. Efficacy and safety of burosumab in children aged 1-4 years with X-linked hypophosphataemia: a multicentre, open-label, phase 2 trial. *Lancet*. (2019) 7:189–99. doi: 10.1016/S2213-8587(18)30338-3

126. Imel EA, Glorieux FH, Whyte MP, Munns CF, Ward LM, Nilsson O, et al. Burosumab versus conventional therapy in children with X-linked hypophosphataemia: a randomised, active-controlled, open-label, phase 3 trial. *Lancet*. (2019) 393:2416–27. doi: 10.1016/S2213-8587(18)30338-3

127. Martín Ramos S, Gil-Calvo M, Roldán V, Castellano Martínez A, Santos F. Positive response to one-year treatment with burosumab in pediatric patients with X-linked hypophosphatemia. *Front Pediatr*. (2020) 8:48. doi: 10.3389/fped.2020.00048

128. Linglart A, Imel EA, Whyte MP, Portale AA, Högler W, Boot AM. Sustained efficacy and safety of burosumab, a monoclonal antibody to FGF23, in children with X-linked hypophosphatemia. *J Clin Endocrinol Metab*. (2022) 107:813–24. doi: 10.1210/clinem/dgab729

129. Namba N, Kubota T, Muroya K, Tanaka H, Kanematsu M, Kojima M, et al. Safety and efficacy of burosumab in pediatric patients with X-linked hypophosphatemia: a phase 3/4 open-label trial. *J Endocr Soc*. (2022) 6:bvac021. doi: 10.1210/jendso/bvac021

130. Paloian NJ, Blaise Nemeth B, Sharafinski M, Modaff P, Steiner RD. Real-world effectiveness of burosumab in children with X-linked hypophosphatemic rickets. *Pediatr Nephrol*. (2022) 37:2667–77. doi: 10.1007/s00467-022-05484-7

131. Ward LM, Glorieux FH, Whyte MP, Munns CF, Portale AA, Högler W, et al. Effect of burosumab compared with conventional therapy on younger vs older children with X-linked hypophosphatemia. *J Clin Endocrinol Metab*. (2022) 107:e3241–53. doi: 10.1210/clinem/dgac296

132. Walker EYX, Lindsay TAJ, Allgrove J, Marlais M, Bockenhauer D, Hayes W. Burosumab in management of X-linked hypophosphataemia: a retrospective cohort study of growth and serum phosphate levels. *Arch Dis Child*. (2023) 108:379–84. doi: 10.1136/archdischild-2022-324962

133. Filler G, Tremblay O, Chen E, Huang SSH, Stein R. Sex differences of burosumab in children with X-linked hypophosphataemic rickets. *Pediatr Nephrol*. (2023) 38:3183–7. doi: 10.1007/s00467-022-05822-9

134. Padidela R, Cheung MS, Saraff V, Dharmaraj P. Clinical guidelines for burosumab in the treatment of XLH in children and adolescents: British paediatric and adolescent bone group recommendations. *Endocr Connect*. (2020) 9:1051–6. doi: 10.1530/EC-20-0291

135. Mughal MZ, Baroncelli GI, de Lucas-Collantes C, Linglart A, Magnolato A, Raimann A, et al. Burosumab for X-linked hypophosphatemia in children and adolescents: opinion based on early experience in seven European countries. *Front Endocrinol*. (2023) 13:1034580. doi: 10.3389/fendo.2022.1034580

136. Bacchetta J, Rothenbuhler A, Gueorguieva I, Kamenicky P, Salles J-P, Briot K, et al. X-linked hypophosphatemia and burosumab: practical clinical points from the French experience. *Joint Bone Spine*. (2021) 88:105208. doi: 10.1016/j.jbspin.2021.105208

137. Imel EA. Burosumab for pediatric X-linked hypophosphatemia. *Curr Osteoporos Rep*. (2021) 19:271–7. doi: 10.1007/s11914-021-00669-9

138. Baroncelli GI, Bertelloni S, Ceccarelli C, Saggese G. Effect of growth hormone treatment on final height, phosphate metabolism, and bone mineral density in children with X-linked hypophosphatemic rickets. *J Pediatr*. (2001) 138:236–43. doi: 10.1067/mpd.2001.108955

139. Huiming Y, Meng M, Chaomin W, Fan Y. Recombinant growth hormone therapy for X-linked hypophosphatemia in children (Review). *Cochrane Database Syst Rev*. (2005) 1:CD004447. doi: 10.1002/14651858.CD004447.pub2

140. Meyerhoff N, Haffner D, Staude H, Wühl E, Marx M, Beetz R, et al. Effects of growth hormone treatment on adult height in severely short children with X-linked hypophosphatemic rickets. *Pediatr Nephrol*. (2018) 33:447–56. doi: 10.1007/s00467-017-3820-3

141. Ertl DA, Le Lorier J, Gleiss A, Trabado S, Bensignor C, Audrain C, et al. Growth pattern in children with X-linked hypophosphatemia treated with burosumab and growth hormone. *Orphanet J Rare Dis*. (2022) 17:412. doi: 10.1186/s13023-022-02562-9

142. Rafaelsen SH, Ræder H, Fagerheim AK, Knappskog P, Carpenter TO, Johansson S, et al. Exome sequencing reveals FAM20C mutations associated with fibroblast growth factor 23– related hypophosphatemia, dental anomalies, and ectopic calcification. *J Bone Miner Res*. (2013) 28:1378–85. doi: 10.1002/jbmr.1850

143. Mameli C, Zichichi G, Mahmood N, Elalaoui SC, Mirza A, Dharmaraj P, et al. Natural history of non-lethal Raine syndrome during childhood. *Orphanet J Rare Dis*. (2020) 15:93. doi: 10.1186/s13023-020-01373-0

144. Hirst L, Abou-Ameira G, Critchlow S. Hypophosphataemic rickets secondary to Raine syndrome: a review of the literature and case reports of three paediatric patients' dental management. *Case Rep Pediatr*. (2021) 6637180. doi: 10.1155/2021/6637180

145. Riminucci M, Collins MT, Fedarko NS, Cherman N, Corsi A, Kenneth E, et al. FGF-23 in fibrous dysplasia of bone and its relationship to renal phosphate wasting. *J Clin Invest*. (2003) 112:683–92. doi: 10.1172/JCI200318399

146. de Castro LF, Ovejero D, Alison M Boyce AM. Mosaic disorders of FGF23 excess: fibrous dysplasia/McCune-Albright syndrome and cutaneous skeletal hypophosphatemia syndrome. *Eur J Endocrinol*. (2020) 182:R83–99. doi: 10.1530/EJE-19-0969

147. Dahir K, Roberts MS, Krolczyk S, Simmons JH. X-linked hypophosphatemia: a new era in management. *J Endocr Soc*. (2020) 4:bvaa151. doi: 10.1210/jendso/bvaa151

148. Bai X, Levental M, Karaplis AC. Burosumab treatment for autosomal recessive hypophosphatemic rickets type 1 (ARHR1). *J Clin Endocrinol Metab*. (2022) 107:2777–83. doi: 10.1210/clinem/dgac433

149. Gladding A, Vivian Szymczuk V, Auble BA, Boyce AM. Burosumab treatment for fibrous dysplasia. *Bone*. (2021) 150:116004. doi: 10.1016/j.bone.2021.116004

150. Sato T, Azuma Y, Tauchi R, Matsushita M, Ozone C. Burosumab treatment for fibrous dysplasia/McCune-Albright syndrome with severe spine deformity. *J Endocrine Soc*. (2022) 6:A160. Abstract. doi: 10.1210/jendso/bvac150.327

151. Tieder M, Modai D, Samuel R, Arie R, Halabe A, Bab I, et al. Hereditary hypophosphatemic rickets with hypercalciuria. *N Engl J Med*. (1985) 312:611–7. doi: 10.1056/NEJM198503073121003

152. Bergwitz C, Miyamoto KI. Hereditary hypophosphatemic rickets with hypercalciuria: pathophysiology, clinical presentation, diagnosis and therapy. *Pflugers Arch*. (2019) 471:149–63. doi: 10.1007/s00424-018-2184-2

153. Bertholet-Thomas A, Tram N, Dubourg L, Lemoine S, Molin A, Bacchetta J. Fluconazole as a new therapeutic tool to manage patients with NPTIIc (SLC34A3) mutation: a case report. *Am J Kidney Dis*. (2019) 73:886–9. doi: 10.1053/j.ajkd.2018.12.026

154. Dreimane D, Chen A, Bergwitz C. Description of a novel SLC34A3.c.671delT mutation causing hereditary hypophosphatemic rickets with hypercalciuria in two adolescent boys and response to recombinant human growth hormone. *Ther Adv Musculoskelet Dis*. (2020) 12. doi: 10.1177/1759720X20912862

155. Filler G, Schott C, Salerno FR, Ens A, McIntyre CW, Díaz Gonzalez de Ferris ME, et al. Growth hormone therapy in HHRH. *Bone Rep*. (2022) 16:101591. doi: 10.1016/j.bonr.2022.101511

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Dual sources of melatonin and evidence for different primary functions

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This article discusses data showing that mammals, including humans, have two sources of melatonin that exhibit different functions. The best-known source of melatonin, herein referred to as Source #1, is the pineal gland. In this organ, melatonin production is circadian with maximal synthesis and release into the blood and cerebrospinal fluid occurring during the night. Of the total amount of melatonin produced in mammals, we speculate that less than 5% is synthesized by the pineal gland. The melatonin rhythm has the primary function of influencing the circadian clock at the level of the suprachiasmatic nucleus (the CSF melatonin) and the clockwork in all peripheral organs (the blood melatonin) via receptor-mediated actions. A second source of melatonin (Source # 2) is from multiple tissues throughout the body, probably being synthesized in the mitochondria of these cells. This constitutes the bulk of the melatonin produced in mammals and is concerned with metabolic regulation. This review emphasizes the action of melatonin from peripheral sources in determining re-dox homeostasis, but it has other critical metabolic effects as well. Extrapineal melatonin synthesis does not exhibit a circadian rhythm and it is not released into the blood but acts locally in its cell of origin and possibly in a paracrine matter on adjacent cells. The factors that control/influence melatonin synthesis at extrapineal sites are unknown. We propose that the concentration of melatonin in these cells is determined by the subcellular redox state and that melatonin synthesis may be inducible under stressful conditions as in plant cells.

KEYWORDS

extrapineal melatonin, circadian rhythms, suprachiasmatic nucleus, mitochondria, redox homeostasis, free radicals, cerebrospinal fluid, cell metabolism

1 Introduction

During their early investigative history, the pineal gland/organ and associated epithalamic structures were morphologically described in detail in many vertebrate species. A significant portion of these publications reported that the outgrowths of the posterodorsal thalamus, i.e., the pineal and the frontal organ (also collectively known as the pineal complex in amphibians), contain photoreceptors much like those in the lateral eyes suggesting they respond directly to light penetrating the area; at that time, the pineal was often referred to as the third eye (1). Further evidence such as the presence of a cartilaginous transparent plate enclosing the occipital fontanel, which allowed the easy penetration of light into the epithalamus, indicated the same as did electrical activity recordings from the light stimulated pineal in species such as the teleost, *Salmo irideus* (2). With the discovery of a genuine dark-dependent specific secretory product (melatonin) in the mammalian pineal gland in the late 1950s, the investigative landscape of the gland dramatically and rapidly changed with developments in the field currently occurring at an almost exponential rate (3).

It is now apparent that melatonin is not exclusively of pineal origin. There are two major endogenous sources of this molecule, herein referred to as Source # 1 (pineal melatonin) and Source # 2 (melatonin produced in extrapineal tissues). These melatonin pools are differentially regulated and, based on current evidence, also have different primary functions. These are concepts elaborated in the current review.

2 Pineal melatonin (Source # 1)

2.1 The backstory

N-acetyl-5-methoxytryptamine, commonly known as melatonin, was isolated and chemically identified in bovine pineal tissue (4, 5). Prior to that, the pineal gland was considered to be evolutionarily vestigial. The impetus for this study was actually published 50 years earlier when it was reported that feeding frog larvae (tadpoles) minced bovine pineal glands caused their skin to lighten dramatically due to the aggregation of the melanin pigment around the nucleus of skin chromatophores (6). Lerner and colleagues (5), since they were dermatologists, surmised that the isolated factor of pineal origin might be useful in treating the human skin disorder known as vitiligo. Unfortunately, after its isolation and when tested in humans, melatonin did not appreciably alter the abnormal skin pigmentation in human melanocytes (7). Shortly after its discovery, the biosynthetic pathway of melatonin from serotonin was identified; thus, it was shown that serotonin is first N-acetylated to form N-acetylserotonin which is then O-methylated resulting in the formation of melatonin (8, 9).

Almost concurrent with the identification of the biosynthetic pathway for melatonin from serotonin, investigators initiated studies related to the effects of the light:dark cycle on pineal melatonin synthesis. These investigations were stimulated by earlier observations showing that pinealocyte morphology changed due to alterations in the photoperiod to which animals were exposed (10, 11). Thus, Wurtman and co-workers (12) exposed rats to continual darkness for 6 days and reported a striking rise in the activity of the melatonin synthesizing enzyme hydroxyindole-O-methyltransferase (HIOMT) (now called serotonin N-acetyltransferase/ASMT) and surmised that pineal melatonin production also was elevated during the dark. While they were correct in their assumption that pineal melatonin synthesis is elevated during darkness, subsequent studies have not shown that HIOMT/ASMT rises appreciably during the daily dark period. Rather, N-acetyltransferase (NAT), which controls the conversion of serotonin to N-acetylserotonin, exhibits a very large nighttime rise in activity and is rate-limiting enzyme in pineal melatonin production (13).

In Figure 1, the enzyme regulating the conversion of serotonin to N-acetylserotonin is listed as AANAT (arylalkylamine N-acetyltransferase). The acronym, NAT, is the more broad-spectrum arylamine N-acetyltransferase that also acetylates serotonin, which allows for the production of melatonin in species that are deficient in AANAT and incorrectly defined as melatonin knock out (14); this enzyme allows for melatonin production in peripheral organs as described by Slominski and colleagues (15). In addition, acetylation of serotonin by the alternative to AANAT was described not only in C57BL/6 mouse but also in humans, rats and hamsters (16–18).

Quay was the first to document that pineal melatonin synthesis was cyclic and that its production was clearly higher at night than during the day (19). Moreover, it was quickly shown that the function of the pineal gland (20) as well as its biosynthetic activity (12) are related to the light:dark cycle perceived by the lateral eyes rather than being dependent on direct photostimulation as had been reported in some amphibians and reptiles (21). This dependency was not unexpected considering the already-described definition of the neural connections between the visual system and the rat pineal gland (22). The circadian rhythm in pineal melatonin synthesis and secretion has now been confirmed in dozens of mammalian species, including in the human (23) and is considered axiomatic (Figure 1).

2.2 Functions of pineal-derived melatonin (Source # 1)

While Lerner and Nordlund failed to show that oral melatonin altered pigment distribution in human melanocytes as with amphibian larvae (7), interest quickly shifted to pineal (melatonin)/reproductive interactions. These studies were performed since even prior to the discovery of melatonin, books authored by Kitay and Altschule (24) and Thieblot and LeBars (25), had hinted at the possibility that pineal function may impact reproductive physiology. Different approaches were taken for these investigations; Wurtman et al. (26) treated rats with melatonin, maintained them under long photoperiods and thereafter examined pubertal development and the estrous cycle, based on the time of vaginal opening and on daily vaginal smears. The results were ambiguous with very minor changes seen in

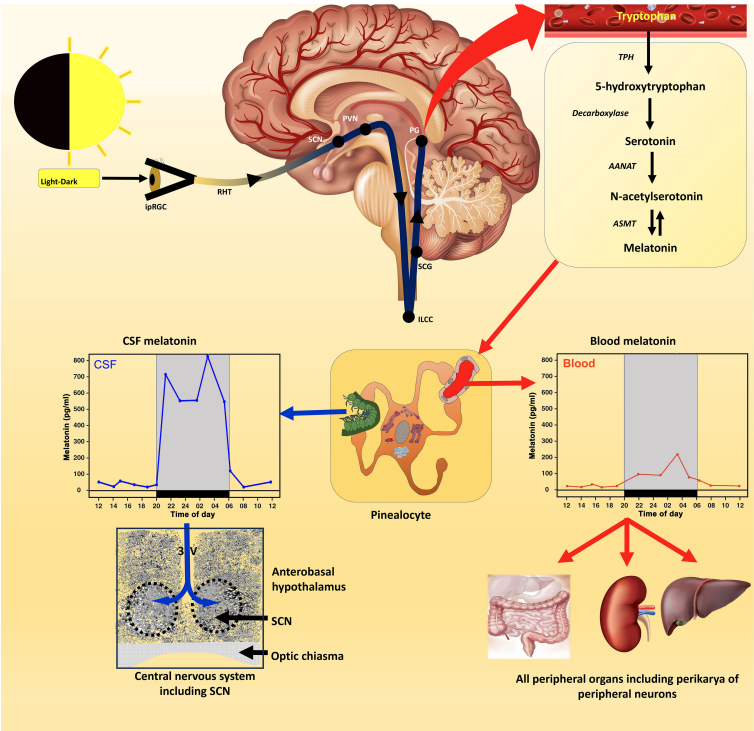

FIGURE 1
The control of melatonin synthesis in the pineal gland (Source # 1) of vertebrates, especially mammals, has been thoroughly investigated from the level of the eyes to the release of melatonin from the gland (top panel); numerous excellent articles and reviews have discussed these subjects which the reader can consult for details. Conventionally, it was thought that the primary route of melatonin secretion was into the perfuse capillary bed in the pineal with nocturnal blood levels exhibiting a measurable rise (middle panel); blood values are typically in the pg/mL range in this fluid. Melatonin is also released into the third ventricular cerebrospinal fluid (CSF) with the rhythm in this fluid exhibiting a much greater amplitude than in the blood. Melatonin diffuses quickly throughout the ventricles and at the base of the third ventricle melatonin presumably has easy access to the master circadian oscillator (suprachiasmatic nucleus; SCN) either due to simple diffusion or transfer via tanycytes. This feedback effect of melatonin on the circadian clock, which is receptor mediated, has an important role in synchronizing circadian processes within the SCN helping to ensure well-regulated 24-hour rhythms throughout the body, e.g., sleep-wake cycle (a primary function of Source # 1 melatonin). The duration of the nocturnal rise in melatonin which is dependent on seasonally changing day/night length also mediates circannual rhythms. The SCN influences circadian rhythms via the visceral/involuntary nervous system (the autonomic nervous system) which innervates smooth and cardiac muscle and many exocrine and endocrine glands. Since all cells are assumed to possess clock genes, the circadian blood melatonin cycle is believed to influence their expression including cells not directly innervated by the autonomic nervous system. In advanced age, the SCN/pineal/melatonin axis deteriorates leading to weaken circadian rhythms which negatively impact disease incidence and general health.

pubertal onset and estrous cycle perturbations. The choice of the rat for this study was not optimal, since its reproductive system is essentially insensitive to pineal removal or to melatonin given that this highly inbred species is not a seasonal breeder (27). By comparison, pineal removal (with the loss of the circadian melatonin rhythm) prevented the dramatic reproductive collapse that occurs in the photosensitive Syrian hamster (*Mesocricetus auratus*) kept under short, winter-type photoperiods; this effect was observed in both male and female hamsters (28, 29). These were the first results to unequivocally document an important role for the pineal gland and melatonin on any aspect of physiology in a mammal. Furthermore, these findings, along with others, led to the now well-established theory that seasonal fluctuations in reproductive capability in photosensitve species are a result of the changing duration of the elevated nocturnal pineal melatonin secretion, which is determined by the annual fluctuations in night length (30, 31). Thus, the melatonin rhythm provides both clock and calendar information (32).

One feature that has complicated the identification of the mechanisms by which the changing melatonin signal regulates annual reproductive changes in seasonally breeding mammals is the fact that in the Syrian hamster (a long day breeder) the reproductive organs are atrophic in the winter which prevents mating, pregnancy and delivery of the young (30). Conversely, in seasonally breeding sheep (a short-day breeder) just the opposite occurs; thus, they are reproductively competent in the short days of the winter but not in the summer (33). Thus, the long duration daily melatonin rise, typically of the short days of the winter, can either inhibit or promote reproductive capability. This suggests that melatonin is neither pro- nor anti-gonadotrophic; rather it is a passive signal of night length which provides time of year information (calendar) (32) with the use of the message being species dependent. A recent review (34) summarizes some of the hypothalamic mechanisms by which melatonin mediates seasonal reproductive changes.

Based on the rhythmic secretion of melatonin from the pineal, the circadian actions of the molecule were quickly investigated and

thousands of reports related to this subject have been published (35). This important research has led to advances in our understanding of the function of the master circadian pacemaker, i.e., the suprachiasmatic nucleus (SCN) (36), which melatonin helps to entrain and to the novel mechanisms of photoreception involved in the regulation of physiological rhythms generally and the pineal melatonin cycle specifically (Figure 1) (37).

The melatonin/circadian rhythm field is massive with many clinical applications already having been proposed or established (38). Many of these studies relate to melatonin's ability to promote sleep (39). It seems likely that there is no system in the organism, either normal or pathological, that avoids the influence of the SCN or the melatonin cycle (40, 41). Clock genes related to these actions are found in essentially every cell (42, 43).

Since the circadian melatonin cycle has been identified in the blood of every mammalian species where it has been examined (with the possible exception of some mice which genetically lack the enzymatic machinery to synthesize melatonin in the pineal gland), it was a reasonable assumption that the synchronizing actions of melatonin on the SCN is a result of the variation in day:night blood melatonin levels (44). Besides releasing melatonin into the circulation in small amounts (usually levels are in the range of pg/mL), melatonin is also discharged directly into the cerebrospinal fluid (CSF) of the third ventricle and is likely an important primary secretory pathway since the nocturnal elevation in this fluid is roughly an order of magnitude greater than that in the blood (Figure 1) (45). Since melatonin in the third ventricular CSF would have easy access to the nearby SCN, it has been suggested that the greater amplitude CSF melatonin cycle is actually responsible for its synchronizing effect at the level of the SCN (46). Under any circumstances, the relationship between melatonin and control of circadian rhythms, and thereby also circannual cycles by the SCN, is indisputable and are major functions of pineal-derived melatonin (Source #1) (30, 47, 48).

While the circadian melatonin rhythm in the CSF may synchronize the activity of the SCN, it seems likely that the blood melatonin cycle regulates the clock genes in peripheral cells (Figure 1) (49) along with neural information that arrives from the SCN via the autonomic nervous system innervation. The ability of the melatonin cycle to adjust circadian biology is also consistent with the observations that the diminished melatonin levels in the aged are associated with generalized chronodisruption (35). It is not known, however, whether the deteriorating melatonin values in the aged interrupt normal SCN physiology or whether the faltering SCN function causes the drop in melatonin secretion since these actions are mutually dependent. Seemingly the major functions of the cyclic production of melatonin by the pineal gland of vertebrates relate to circadian biology.

Surgical removal of the pineal gland in mammals is usually associated with essentially an absence of blood melatonin levels with no discernible rhythm. Pinealectomy in some poikilothermic vertebrates, however, does not result in a loss of the circadian blood melatonin cycle (50). Also, in these species, the melatonin rhythm may be dictated by day:night ambient temperature variations. Melatonin is associated with the pineal gland only in vertebrates since they are the only species that have this organ; still, this indoleamine is found (Source #2) throughout the animal and plant kingdoms including protists and in plants, all species that lack a pineal gland and have no equivalent homolog. There are also some vertebrates that lack a morphological discernible pineal gland, but still exhibit a light/dark-related low amplitude blood melatonin cycle, e.g., the alligator (*Alligator mississippiensis*) (51), and other members of the subclass of Archoasurian reptiles. The authors surmised that the melatonin cycle in these species is probably not involved in circadian regulation; moreover, they felt this rhythm does not originate from pineal tissue, i.e., rather being of extrapineal origin, since this structure is morphological absent. It seems more likely, however, that these species have functional pinealocytes diffusely distributed in their epithalamus that are not organized into an identifiable discrete gland.

3 Extrapineal melatonin (Source # 2)

3.1 The backstory

Within a year after the discovery of melatonin in the pineal gland (5), the same group of investigators also found melatonin in the sciatic nerves of humans and in other mammalian peripheral nerves (52). While they did not speculate on its origin since they did not examine its local synthesis, most likely they thought it was pineal-derived melatonin taken up from the blood.

Since then, melatonin has been identified in many cells not associated with the pineal gland; this is generally referred to as extrapineal melatonin (Source#2) (53, 54). The amount of melatonin found outside the pineal gland is massive in comparison with what presumably could be produced in this small neural outgrowth where its synthesis only occurs during the daily dark period. This also becomes highly relevant in animals living at extremely high latitudes where persistent darkness for long periods totally eliminates the blood melatonin rhythm, sometimes for multiple months of the year (55). If the total melatonin load in these species was derived exclusively from the pineal gland, they would be devoid of all melatonin for a significant portion of each year. Also noteworthy is that non-vertebrate species, that is, invertebrates, protists and plants, also contain melatonin, sometimes in much higher concentrations than in vertebrates (56–58). It seems unlikely that the cells of millions of species that lack any semblance of a pineal gland would synthesize melatonin, while vertebrate non-pineal cells would not do so.

The proposed evolution of melatonin also predicts that melatonin would be produced in many cells/organs in addition to the pineal gland (59, 60). As currently theorized, melatonin evolved 2.5 to 2.0 billion years ago (bya) in bacteria at a time when eukaryotes did not yet exist, with its initial function being that of a reactive oxygen species (ROS) scavenger (61). Its evolution may have occurred in association with the Great Oxidation Event (2.5-2.0 bya) when the Earth's atmospheric concentrations of oxygen rose profoundly because of its release from the photosynthesizing microbes, cyanobacteria (Figure 2) (62, 63). Although the photosynthetic prokaryotes, cyanobacteria, did not possess chloroplasts, they had membranous photosynthesizing pigments

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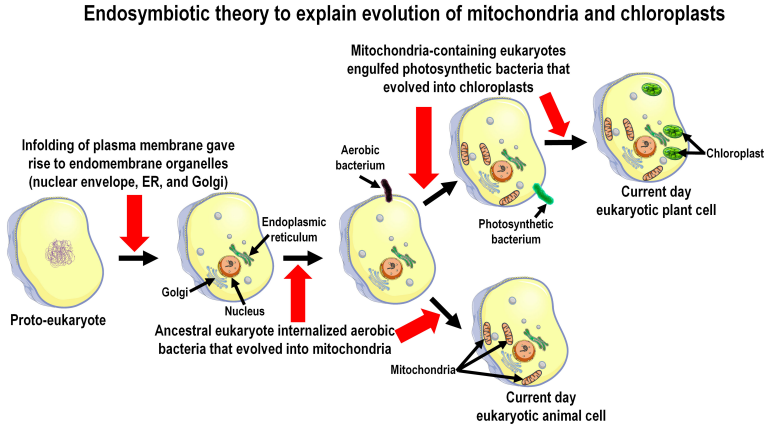

FIGURE 2
The presence of melatonin in the mitochondria and chloroplasts of present-day species is consistent with the endosymbiotic theory which explains the derivation of these organelles, which is diagrammatically summarized in this figure. Proto-prokaryotic organisms (bacteria) are estimated to have evolved perhaps as early as 3.7 to 3.5 billion years ago; over the next billion and a half years these cells developed intracellular organelles resulting in the formation of primitive eukaryotes. Melatonin synthesizing capacity in prokaryotes predictably evolved during the geologic period referred to as the Great Oxidation Event (2.5 to 2.0 billion years ago) which necessitated organisms to develop antioxidants to evade the toxicity of oxygen-based derivatives. During their evolution eukaryotes engulfed/phagocytized prokaryotes for food. Over time these engulfed organisms, i.e., either protobacteria (forming mitochondria) or photosynthesizing cyanobacteria (forming chloroplasts), developed a symbiotic relationship with their host cells. The host cells took advantage of the energy producing network and the melatonin-forming capacity of the engulfed prokaryotes and, because of these functions were beneficial, both were preserved in all eukaryotic cells that exist today.

which could capture and use solar energy for photosynthesis. Due to the toxicity of the oxygen-based derivatives (ROS), the need for protective antioxidants increased, presumably resulting in the evolution of melatonin and other ROS neutralizing or metabolizing species (64). The evolution of melatonin presumably occurred concurrently in both photosynthesizing cyanobacteria and in non-photosynthesizing bacteria, e.g., α-proteobacteria, of which multiple types share a common ancestor (65).

With the arrival of primitive eukaryotes, they internalized melatonin-producing bacteria as food or as an energy source. The phagocytized non-photosynthetic bacteria (symbionts) over eons evolved into mitochondria while the engulfed photosynthesizing bacteria became chloroplasts (known as the well-established endosymbiotic theory) of the early eukaryotes and during this process the melatonin-synthesizing capacity of the bacteria-derived organelles, both chloroplasts and mitochondria, was retained (Figure 2). Moreover, since all present-day eukaryotic cells (with few exceptions) contain mitochondria, chloroplasts, or both, they continue to be a source of melatonin (see below). The pineal gland, which is only found in vertebrates (which did not appear until the Cambrian explosion about 520 million years ago; mya), is a rather recently evolved organ for melatonin synthesis. Thus, melatonin likely existed for millions of years before the pineal gland even evolved. When melatonin receptors first appeared has not been thoroughly investigated but is surmised to have occurred during the Triassic Period (250 mya) (66), so the functions of melatonin (and its metabolites) prior to that time were receptor-independent, e.g., they functioned in the modulation of metabolic processes without an intervening receptor including as a direct ROS scavenger, etc. (61). Both non-receptor-mediated and receptor-dependent functions of melatonin are retained in present day vertebrate species (67).

Numerous papers documenting the presence of melatonin in invertebrates and in protists, including algae, which are not classified as plants although most algae species have the capacity of photosynthesis for energy production. The findings of these reports prompted investigations into examining the possible presence of melatonin in photosynthetic land plants. Three reports were published in 1995 by independent groups of investigators that simultaneously showed unequivocally that the plants tested contain melatonin. In these studies, multiple techniques were used to identify melatonin in the plant species examined (68–70); shortly thereafter, it was reported that the melatonin concentration in the seeds of edible plants varies widely (71). Furthermore, melatonin has been identified in all plant organs: leaves, stems, roots, flowers, etc. (72), some of which contain cells capable of photosynthesis and others that do not. Since melatonin produced by plant cells cannot be quickly circulated/transferred among plant organs, it is likely that all cells generate this indoleamine, i.e., a single plant tissue does not produce melatonin which is then distributed to other plant appendages.

Surgical removal of the pineal gland in mammals is always associated with essentially an absence of blood melatonin levels with no discernible rhythm. Melatonin is associated with the pineal gland only in vertebrates since they are the only species that have this organ; but the indoleamine is found in invertebrates including protists and in plants, all species that lack a pineal gland and have no pineal homolog.

3.2 The role of extrapineal melatonin (Source # 2): regulation of redox homeostasis

Studies on the actions of melatonin in relation to circadian biology continue to be intensively investigated; this action clearly

involves the cyclic production and release of melatonin from the pineal gland (Source #1). More recently, other investigations have proven that the actions of melatonin far exceed those that influence clock functions. Mounting evidence in recent years supports the extensive interaction between the circadian and redox systems. Such a relationship is not surprising because most diurnal or nocturnal organisms display daily oscillations in energy intake, locomotor activity, and exposure to exogenous and internally generated oxidants (73). The transcriptional clock controls the levels of many antioxidant proteins and redox-active cofactors. Conversely, the cellular redox state has been shown to feed back to the transcriptional oscillator via redox-sensitive transcription factors and enzymes. Thus, these intrinsic clocks are thought to have co-evolved with cellular redox regulation (73, 74).

The discovery and repeated confirmation of melatonin as an oxygen-based free radical scavenger and antioxidant represents a paradigm shift (61, 75–82), especially since this applied to melatonin at both the pinealocyte level, and to extrapineal cells (Source # 2). This provides all cells additional protection against the continual bombardment and potential molecular destruction caused by ROS as well as by reactive nitrogen species (RNS) (83–85). Even though melatonin and its metabolites are highly efficient radical detoxifiers (86–88), it is apparent that the small quantity of melatonin released from the pineal gland on a nightly basis would not be sufficient to combat or neutralize the total radical free load that an entire organism generates every day. Thus, if melatonin does function as a system-wide antioxidant, sources of melatonin other than the pineal gland would be necessary. Obviously, this is even more apparent in invertebrates, protists, and plants that have no pineal gland. This problem was solved when melatonin was identified in many peripheral organs (53, 89, 90) and even more so when it is located in the mitochondria of these cells as has been shown (91).

Not only is melatonin produced by peripheral cells, but importantly it is likely generated specifically in mitochondria (Figure 3) (59, 92, 93); this is critical since these organelles are major contributors to free radical generation. The presence of melatonin in these organelles is likely of special importance when there is a rapid deluge of newly produced radicals such as occurs during exposure to ionizing radiation (94), injurious chemicals such as paraquat (95), or toxic drugs such as including chemotherapies (96). During these precarious situations it is essential to have a multifaceted antioxidant in mitochondria to quickly neutralize the highly reactive ROS/RNS to avoid functional deterioration of these critically-important organelles. Moreover, if melatonin is inducible in animal cell mitochondria, as we suspect it is, it would be even more beneficial in combatting the acute free radical toxicity of hazardous processes or toxins. The likely upregulation of melatonin synthesis in stressed mitochondria is also strongly supported by abundant data showing that plant cells exhibit a rapid compensatory upregulated melatonin production when they are exposed to radical-mediated stresses resulting from multiple events, e.g., cold or hot ambient temperatures (97, 98), increased salinity (99) or chemical exposure (100), drought (101), etc. That melatonin scavenges radicals in mitochondria is well documented (102).

Locally produced melatonin also protects the intramitochondrial environment from oxidative stress since in addition to acting as a direct radical scavenger it also stimulates enzymes, i.e., superoxide dismutase 2 (SOD2) and glutathione peroxidase (GPx), that remove ROS from the mitochondrial matrix (103). The elevated SOD2 activity is achieved as a result of an upregulation of sirtuin 3 (SIRT3), one member of a family of epigenetic enzymes located primarily in the mitochondrial matrix that regulates a variety of metabolic processes (104, 105). Also, SIRT3 is upregulated by oxidative stress in both mammalian as well as in invertebrate neurons which in turn stimulates the detoxification of ROS due to its capacity to promote the activity of SOD (106). This latter observation suggests the possibility that oxidative stress is consequential in the reported compensatory rise in subcellular melatonin production, as commonly observed in plants and which has also been reported in algae (97, 107), which in turn upregulates the SIRT3/FOXO/SOD2 pathway, thereby helping to maintain oxidative homeostasis under elevated oxidative stress conditions. This would obviously be a very important function of Source # 2 melatonin in animals.

The normal nocturnal rise in pineal melatonin and its release (Source # 1) are mediated due to the interaction of postganglionic neuron norepinephrine (NE) with specific receptors on the pinealocyte membrane (108). Pineal melatonin production is not inducible; when stress-mediated circulating NE rise during the day, the nerve endings in the pineal gland reportedly act as a sink to sequester high blood NE levels (109, 110). Thus, pineal melatonin synthesis is not elevated as a result of systemic stress which causes the discharge of catecholamines from other organs, e.g., the adrenal medulla. Moreover, pineal melatonin synthesis is not consistently exaggerated by systemic free radical excesses.

In comparison to what is known about Source # 1 melatonin regulation, information about the control of extrapineal/mitochondrial melatonin (Source # 2) production is negligible. It is unknown whether circulating NE influences mitochondrial melatonin synthesis in peripheral organs since melatonin is not usually released from these cells and as a result it would not be reflected in blood melatonin concentrations. This suggests that the mechanisms for the regulation of melatonin stimulation probably differ between these sites.

A recent publication alluded to the possibility that non-visible near infrared radiation (NIR) may be a factor in the regulation of melatonin in peripheral cells. NIR has high penetrability through the skin and into some deeper structures (111). Melatonin is known to be synthesized in dermal and epidermal cells (112, 113) where it has critical functions in the protection of the skin from ROS/RNS induced by ultraviolet radiation exposure or chemical toxins (114). During exposure to the sun, the skin is exposed to both UVR and NIR; these electromagnetic radiations theoretically have contrasting actions in skin cells. Thus, UVR damages dermal and epidermal cells because it generates destructive free radicals while NIR promotes melatonin production in the same cells to combat the associated oxidative stress (115, 116). The beneficial effects of NIR on human health are well recognized, an action that could involve its capacity to promote melatonin production (117). The use of NIR therapy is referred to as photobiomodulation and is widely used for the treatment of a variety of diseases (116).

Melatonin of Source # 2 means this multifunctional molecule is always available, during both the day and at night. Historically, it

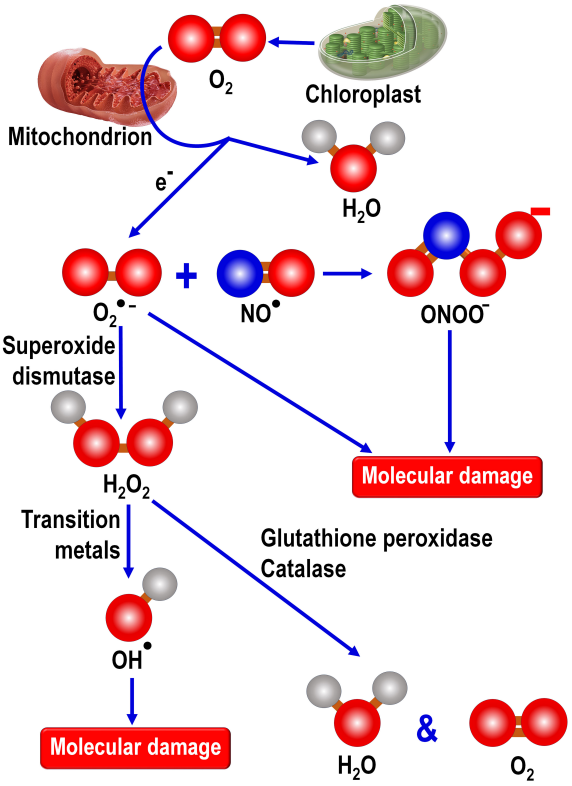

FIGURE 3
During photosynthesis by land plants, chloroplasts released oxygen as a byproduct of photosynthesis. During the Great Oxidation Event more than 2 billion years ago, atmospheric oxygen gradually rose as a consequence of oxygen released by photosynthetic prokaryotes, cyanobacteria. Early in evolution, engulfed cyanobacteria gave rise to chloroplasts in primitive photosynthesizing eukaryotes which, to this day, contribute highly significantly to atmospheric oxygen levels along with that derived from land plants. The metabolism of oxygen by mitochondria, which are present in essentially all animal and plant eukaryotes, generates the superoxide anion ($O_2^{\bullet -}$) radical which is the precursor of a number of other toxic oxygen-based and nitrogen-based products. $O2^{\bullet -}$ couples with nitric oxide ($NO^{\bullet}$) to form peroxynitrite ($ONOO^-$), a powerful oxidizing agent. The enzymatic dismutation of $O_2^{\bullet -}$ forms hydrogen peroxide (H_2O_2), a non-radical product that exhibits high diffusibility and which can be metabolically degraded by either glutathione peroxidase or catalase. In the presence of a transition metal, often Fe^{2+}, H_2O_2 is converted to the highly reactive and destructive hydroxy radical ($OH^{\bullet}$). Cells have no means to enzymatically remove the $OH^{\bullet}$ and its extremely short half-life means it must be formed in the vicinity of a radical scavenger if it is to be incapacitated before it inflicts molecular damage. Thus, since $OH^{\bullet}$ is formed in high concentrations in mitochondria, it is important that melatonin also be situated in this organelle to be available for scavenging this reactant. As a direct free radical scavenger, melatonin reportedly neutralizes the $O_2^{\bullet -}$, H_2O_2, $OH^{\bullet}$, $NO^{\bullet}$ and $ONOO^-$. Additionally, melatonin stimulates the radical metabolizing enzymes superoxide dismutase, glutathione peroxidase and catalase. It also enhances the concentration of another important antioxidant, glutathione, by stimulating the rate limiting enzyme, glutamine cysteine ligase, required for glutathione synthesis.

was presumed by many that the pineal gland was the only or chief source of melatonin. Zhao and colleagues (60) recently proposed that in reality even in those organisms that have a pineal gland less than 5% derives from this organ. If the pineal was the exclusive source of melatonin, which it is obviously not, the functions of this essential agent would be absent during the daily light period when, at least in diurnally-active animals, oxidative stress is highest because of UV exposure, psychological and physical stress, etc. Source # 2 melatonin ensures that it is available during the day when humans are most likely to experience free radical damage. Moreover, if the production of melatonin in peripheral cells is definitively proven to be upregulated by NIR exposure (or any other stimulus) it could be critical in disease prevention and for deferred aging. Finally, in addition to melatonin being available from the pineal and extrapineal tissues, its intake in the diet and contribution made by microbiota may also prove to be significant (116).

While melatonin is well-documented to directly neutralize ROS and RNS, it does not function in this regard without assistance. When melatonin donates and electron to inactivate a radical species, it is transformed into cyclic 3-hydroxymelatonin, which is also a radical scavenger. Figure 4 illustrates the additional melatonin derivatives that function in scavenging radical species in what is referred to as the melatonin's antioxidant cascade (118). Some of these derivatives and others are better scavengers than melatonin itself (87, 119, 120). Finally, melatonin chelates transition metals to reduce the formation of the hydroxyl radical during the Fenton reaction or Haber-Weiss reactions (Figure 4) (86).

4 Discussion

Melatonin is phylogenetically an ancient molecule which, during billions of years of evolution, has been repurposed for a variety of functions. The presently available evidence suggests melatonin first appeared in prokaryotes. There-after, due to an endosymbiotic association that the prokaryotes established with primitive

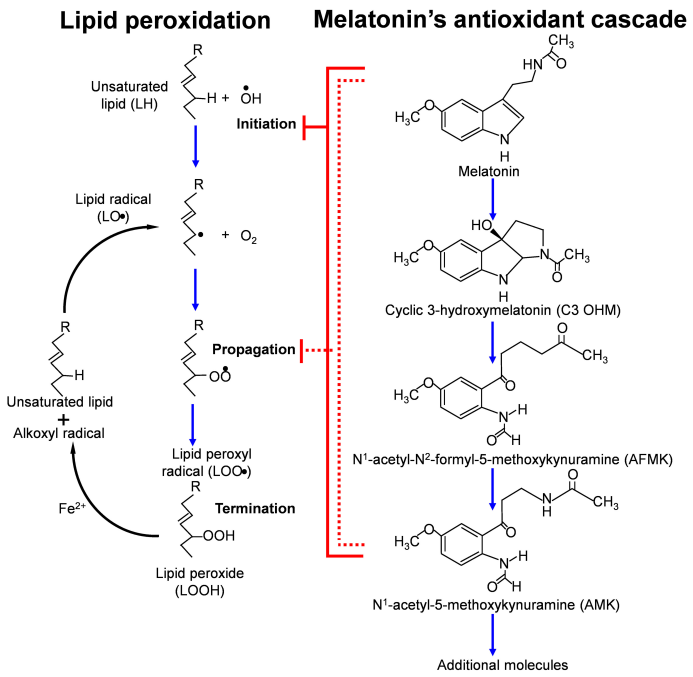

FIGURE 4
This figure summarizes some of the metabolites that are formed (in what is referred to as the antioxidant cascade) when melatonin functions as a direct free radical scavenger while inhibiting the initiation and propagation of lipid peroxidation. The metabolites of melatonin also function to neutralized ROS/RNS with some of them being more efficient radical scavengers that melatonin itself. Melatonin reduces the peroxidation of unsaturated lipids by scavenging the $OH^{\bullet}$ and other radicals that initiate the breakdown of easily oxidizable lipids. As indicated, the metabolites also interrupt the propagation of lipid peroxidation by neutralizing the lipid peroxyl radical.

eukaryotes after their internalization, they evolved into mitochondria. Melatonin's use as an antioxidant possibly was a result of the Great Oxidative Event during which atmospheric oxygen rose profoundly necessitating the need for molecules that were capable of neutralizing toxic oxygen derivatives, i.e., free radicals. High free radical production occurred in primitive animal eukaryotic cells when they began using oxygen as a basis of their metabolism. Additionally, photosynthetic eukaryotes generate oxygen as a byproduct of photosynthesis that is converted to toxic metabolites under abiotic stress conditions which could damage biomolecules of the photosynthetic apparatus. Over subsequent evolutionary periods to the present day, melatonin retained its ability to detoxify reactive ROS. Moreover, since this function was inherited from engulfed prokaryotes which became mitochondria, the melatonin synthesizing capacity was presumably retained in these organelles to the present day. This ideally positioned melatonin as a radical scavenger since the process of oxidative phosphorylation in mitochondria is the site of the production of the superoxide anion radical, the precursor of all other destructive ROS as well as reactive nitrogen species (RNS) (Figure 3).

Many peripheral, perhaps all, cells in tissues of both animal and plant eukaryotic organisms produce melatonin likely as a protection against biomolecular destruction by ROS/RNS as well as other functions. The amount of melatonin synthesized in peripheral cells is presumed to be cell specific and stress de-pendent, i.e., potentially upregulated in animal cells as has been well documented in plants (121). The skin is an example in which proper production of melatonin is linked to protection against solar radiation (10, 113). Mitochondria as the specific site of intracellular melatonin production is generally supported by the published data and is consistent with the evolution of these organelles, which were derived from prokaryotes that presumably had the capacity of melatonin synthesis (Figure 2). In addition to the rationale related to evolution of these organelles, compelling arguments for the association of melatonin with mitochondria come from the elegant work of Suofu and colleagues (93) and a publication by He et al. (122). In the latter study, the authors isolated the mitochondria from mouse oocytes, a cell previously shown to synthesize melatonin (123). When these mitochondria were incubated with serotonin as a substrate, they time-dependently generated melatonin; serotonin is a necessary precursor in the melatonin synthetic pathway (110). Conversely, when the oocyte mitochondria were incubated in the absence of serotonin, they failed to produce melatonin. These findings, considered in conjunction with the known contribution of oocyte mitochondria to every cell in vertebrate organisms, support the conclusion that the melatonin synthesizing ability of mitochondria in all cells has been retained to the present day (60). Also, melatonin metabolism within mitochondria can play an important role in their metabolic functions and regulation of cellular phenotype as illustrated in skin cells (124, 125).

Within which mitochondrial compartment melatonin is produced is still under debate. An early immunocytochemical

investigation suggested this occurred in the intramembrane space (126). The results of Suofu et al. (93) showed, however, that removal of the outer mitochondrial membrane, using digitoxin, while leaving the inner mitochondrial membrane intact, did not impact the concentration of melatonin in these organelles indicating that it is located in the matrix. Similar to He and colleagues (122), they confirmed that isolated neural mitochondria synthesized melatonin from its deuterated precursor. Despite the currently available data, the possibility that melatonin is produced in other subcellular organelles in addition to mitochondria cannot be precluded (56) and there is the possibility some cells have lost this capacity.

An interaction between Source # 1 and Source # 2 melatonin has been identified and thoroughly investigated by one group of scientists in what is defined as the immune-pineal axis (127). What this group has found is the nocturnal melatonin surge is suppressed by proinflammatory cytokines while simultaneously inducing melatonin synthesis in bone marrow and spleen macrophages (128). This switch in the primary site of melatonin synthesis relies on NF-κB activation. These highly mechanistic studies indicate that interleukin-10 (IL-10) levels modulate downstream immune responses which are operative in impacting both Source # 1 (pineal) and Source 2 (extrapineal) melatonin concentrations (89). The alterations in melatonin synthesis are predictably essential in modulating the immune response with the locally-synthesized melatonin by immune-competent cells exerting anti-inflammatory actions at the site of infection (129); the inhibitory actions of melatonin on inflammation have been documented in many studies (130). These intriguing results likely have importance in explaining the efficacy of melatonin as an anti-inflammatory agent and they deserve more intensive investigation.

In the current survey, only the antioxidative actions of peripherally generated melatonin were discussed in detail. Free radical-mediated oxidative damage is a component of many diseases so preventing this damage, which melatonin is highly effective in doing, may be a means by which this important molecule preserves general heath and reduces pathologies. Other disease conditions in which melatonin may have utility as a treatment include cancer, cardiovascular disease, neurodegeneration, sepsis, drug toxicity, and many others. Literature searches will uncover extensive reviews on each of these subjects in reference to melatonin. Melatonin from both Source # 1 and Source # 2 is critical for health maintenance. Moreover, in addition to these two sources of melatonin, it can also be obtained in the diet and supplied by microbiota (131, 132).

Author contributions

RR: Conceptualization, Data curation, Project administration, Supervision, Validation, Visualization, Writing – original draft. RS: Project administration, Software, Visualization, Writing – review & editing. DT: Data curation, Writing – review & editing. LC: Data curation, Writing – review & editing. Dd: Data curation, Writing – review & editing. AS: Data curation, Funding acquisition, Investigation, Validation, Writing – review & editing. KS: Data curation, Funding acquisition, Investigation, Writing – review & editing. KK: Data curation, Funding acquisition, Investigation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Eakin RM, Westfall JA. Fine structure of the retina in the reptilian third eye. *J Biophys Biochem Cytol.* (1959) 6:133–4. doi: 10.1083/jcb.6.1.133

2. Dodt E. Photosensitivity of the pineal organ in the teleost, Salmo irideus (Gibbons). *Experientia.* (1963) 19:642–3. doi: 10.1007/BF02151295

3. López-Muñoz FJ, Povedano-Montero FJ, Romero A, Egea J, Álamo C. The crossroads of melatonin: bibliometric analysis and mapping of global scientific research. *Melatonin Res.* (2021) 4:152–72. doi: 10.32794/mr1250088

4. Lerner AB, Case JD, Heinzelman RV. Structure of melatonin. *J Am Chem Soc.* (1959) 81:6084–5. doi: 10.1021/ja01531a060

5. Lerner AB, Case JD, Takahashi Y, Lee TH, Mori W. Isolation of melatonin: the pineal gland factor that lightens melanocytes. *J Am Chem Soc.* (1958) 80:2587–. doi: 10.1021/ja01543a060

6. Mccord CP, Allen FP. Evidences associating pineal gland function with alterations in pigmentation. *J Exp Zool.* (2017) 23:207–24. doi: 10.1002/jez.1400230108

7. Lerner A, Nordlund J. Comment. Administration of melatonin to human subjects. *Front Pineal Physiol.* (1975) 7:42–3.

8. Axelrod J, Weissbach H. Enzymatic O-methylation of N-acetylserotonin to melatonin. *Science.* (1960) 131:1312. doi: 10.1126/science.131.3409.1312

9. Weissbach H, Redfield BG, Axelrod J. Biosynthesis of melatonin: enzymic conversion of serotonin to N-acetylserotonin. *Biochim Biophys Acta*. (1960) 43:352–3. doi: 10.1016/0006-3002(60)90453-4

10. Quay W. Volumetric and cytologic variation in the pineal body of Peromyscus leucopus (Rodentia) with respect to sex, captivity and day-length. *J Morphol*. (1956) 98:471–95. doi: 10.1002/jmor.1050980306

11. Roth WD, Wurtman RJ, Altschule MD. Morphologic changes in the pineal parenchyma cells of rats exposed to continuous light or darkness. *Endocrinology*. (1962) 71:888–92. doi: 10.1210/endo-71-6-888

12. Wurtman RJ, Axelrod J, Phillips LS. Melatonin synthesis in the pineal gland: control by light. *Science*. (1963) 142:1071–3. doi: 10.1126/science.142.3595.1071

13. Klein DC. Arylalkylamine N-acetyltransferase: "The timezyme". *J Biol Chem*. (2007) 282:4233–7. doi: 10.1074/jbc.R600036200

14. Roseboom PH, Namboodiri MA, Zimonjic DB, Popescu NC, Rodriguez IR, Gastel JA, et al. Natural melatonin 'Knockdown' in C57bl/6j mice: rare mechanism truncates serotonin N-acetyltransferase. *Brain Res Mol Brain Res*. (1998) 63:189–97. doi: 10.1016/s0169-328x(98)00273-3

15. Slominski A, Pisarchik A, Semak I, Sweatman T, Wortsman J. Characterization of the serotoninergic system in the C57bl/6 mouse skin. *Eur J Biochem*. (2003) 270:3335–44. doi: 10.1046/j.1432-1033.2003.03708.x

16. Semak I, Korik E, Naumova M, Wortsman J, Slominski A. Serotonin metabolism in rat skin: characterization by liquid chromatography-mass spectrometry. *Arch Biochem Biophys*. (2004) 421:61–6. doi: 10.1016/j.abb.2003.08.036

17. Slominski A, Pisarchik A, Semak I, Sweatman T, Szczesniewski A, Wortsman J. Serotoninergic system in hamster skin. *J Invest Dermatol*. (2002) 119:934–42. doi: 10.1046/j.1523-1747.2002.00156.x

18. Slominski AT, Kim TK, Kleszczynski K, Semak I, Janjetovic Z, Sweatman T, et al. Characterization of serotonin and N-acetylserotonin systems in the human epidermis and skin cells. *J Pineal Res*. (2020) 68:e12626. doi: 10.1111/jpi.12626

19. Quay WB. Circadian and estrous rhythms in pineal melatonin and 5-hydroxy indole-3-acetic acid. *Proc Soc Exp Biol Med*. (1964) 115:710–3. doi: 10.3181/00379727-115-29014

20. Reiter RJ, Hester RJ. Interrelationships of the pineal gland, the superior cervical ganglia and the photoperiod in the regulation of the endocrine systems of hamsters. *Endocrinology*. (1966) 79:1168–70. doi: 10.1210/endo-79-6-1168

21. Oksche A, Harnack Mv. Elektronenmikroskopische untersuchungen am stirnorgan (Frontalorgan, epiphysenendblase) vonrana temporaria undrana esculenta. *Naturwissenschaften*. (1962) 49:429–30. doi: 10.1007/BF00624428

22. Kappers JA. [Innervation of the cerebral epiphysis in albino rats]. *Acta Neuroveg (Wien)*. (1961) 23:111–4. doi: 10.1007/BF01243962

23. Vaughan GM, Pelham RW, Pang SF, Loughlin LL, Wilson KM, Sandock KL, et al. Nocturnal elevation of plasma melatonin and urinary 5-hydroxyindoleacetic acid in young men: attempts at modification by brief changes in environmental lighting and sleep and by autonomic drugs. *J Clin Endocrinol Metab*. (1976) 42:752–64. doi: 10.1210/jcem-42-4-752

24. Kitay JI, Altschule MD. The pineal gland. A review of the physiologic literature. *Pineal Gland*. (1954).

25. Thiéblot L, Le Bars H. La glande pinéale ou épiphyse: anatomie, histologie, physiologie, clinique. (1955)

26. Wurtman RJ, Axelrod J, Chu EW. Melatonin, a pineal substance: effect on the rat ovary. *Science*. (1963) 141:277–8. doi: 10.1126/science.141.3577.277

27. Wragg LE. Effects of pinealectomy in the newborn female rat. *Am J Anat*. (1967) 120:391–402. doi: 10.1002/aja.1001200302

28. Hoffman RA, Reiter RJ. Pineal gland: influence on gonads of male hamsters. *Science*. (1965) 148:1609–11. doi: 10.1126/science.148.3677.1609

29. Hoffman RA, Reiter RJ. Responses of some endocrine organs of female hamsters to pinealectomy and light. *Life Sci*. (1966) 5:1147–51. doi: 10.1016/0024-3205(66)90099-3

30. Reiter RJ. Pineal control of a seasonal reproductive rhythm in male golden hamsters exposed to natural daylight and temperature. *Endocrinology*. (1973) 92:423–30. doi: 10.1210/endo-92-2-423

31. Thorpe PA, Herbert J. Studies on the duration of the breeding season and photorefractoriness in female ferrets pinealectomized or treated with melatonin. *J Endocrinol*. (1976) 70:255–62. doi: 10.1677/joe.0.0700255

32. Reiter RJ. The melatonin rhythm: both a clock and a calendar. *Experientia*. (1993) 49:654–64. doi: 10.1007/BF01923947

33. Lincoln GA, Short RV. Seasonal breeding: nature's contraceptive. *Recent Prog Horm Res*. (1980) 36:1–52. doi: 10.1016/b978-0-12-571136-4.50007-3

34. Korf HW. Signaling pathways to and from the hypophysial Pars tuberalis, an important center for the control of seasonal rhythms. *Gen Comp Endocrinol*. (2018) 258:236–43. doi: 10.1016/j.ygcen.2017.05.011

35. Verma AK, Khan MI, Ashfaq F, Rizvi SI. Crosstalk between aging, circadian rhythm, and melatonin. *Rejuv Res*. (2023) 26:229–41. doi: 10.1089/rej.2023.0047

36. Davidson AJ, Beckner D, Bonnefont X. A journey in the brain's clock: *in vivo* veritas? *Biol (Basel)*. (2023) 12(8):1136. doi: 10.3390/biology12081136

37. Robertson-Dixon I, Murphy MJ, Crewther SG, Riddell N. The influence of light wavelength on human Hpa axis rhythms: A systematic review. *Life (Basel)*. (2023) 13 (10):1968. doi: 10.3390/life13101968

38. de Leeuw M, Verhoeve SI, van der Wee NJA, van Hemert AM, Vreugdenhil E, Coomans CP. The role of the circadian system in the etiology of depression. *Neurosci Biobehav Rev*. (2023) 153:105383. doi: 10.1016/j.neubiorev.2023.105383

39. Sletten TL, Weaver MD, Foster RG, Gozal D, Klerman EB, Rajaratnam SMW, et al. The importance of sleep regularity: A consensus statement of the national sleep foundation sleep timing and variability panel. *Sleep Health*. (2023) 9:801–20. doi: 10.1016/j.sleh.2023.07.016

40. Schettini MAS, Passos R, Koike BDV. Shift work and metabolic syndrome updates: A systematic review. *Sleep Sci*. (2023) 16:237–47. doi: 10.1055/s-0043-1770798

41. Zhu H, Chen J, Wen Z, Li J, Yu Q, Liao W, et al. The role of circadian clock genes in colorectal carcinoma: novel insights into regulatory mechanism and implications in clinical therapy. *Life Sci*. (2023) 333:122145. doi: 10.1016/j.lfs.2023.122145

42. BaHammam AS, Pirzada A. Timing matters: the interplay between early mealtime, circadian rhythms, gene expression, circadian hormones, and metabolism-a narrative review. *Clocks Sleep*. (2023) 5:507–35. doi: 10.3390/clockssleep5030034

43. Patton AP, Hastings MH. The mammalian circadian time-keeping system. *J Huntingtons Dis*. (2023) 12:91–104. doi: 10.3233/JHD-230571

44. Pfeffer M, von Gall C, Wicht H, Korf HW. The role of the melatoninergic system in circadian and seasonal rhythms-insights from different mouse strains. *Front Physiol*. (2022) 13:883637. doi: 10.3389/fphys.2022.883637

45. Legros C, Chesneau D, Boutin JA, Barc C, Malpaux B. Melatonin from Cerebrospinal Fluid but Not from Blood Reaches Sheep Cerebral Tissues under Physiological Conditions. *J Neuroendocrinol*. (2014) 26:151–63. doi: 10.1111/jne.12134

46. Reiter RJ, Sharma R, Rosales-Corral S, de Mange J, Phillips WT, Tan DX, et al. Melatonin in ventricular and subarachnoid cerebrospinal fluid: its function in the neural glymphatic network and biological significance for neurocognitive health. *Biochem Biophys Res Commun*. (2022) 605:70–81. doi: 10.1016/j.bbrc.2022.03.025

47. Imai N. Molecular and cellular neurobiology of circadian and circannual rhythms in migraine: A narrative review. *Int J Mol Sci*. (2023) 24(12):10092. doi: 10.3390/ijms241210092

48. Yang Y, Chu MX, Liu QY. The mechanism of circadian clock and its influence on animal circannual rhythm. *Yi Chuan*. (2023) 45:409–24. doi: 10.16288/j.yczz.23-008

49. Acuna-Castroviejo D, Rahim I, Acuna-Fernandez C, Fernandez-Ortiz M, Solera-Marin J, Sayed RKA, et al. Melatonin, clock genes and mitochondria in sepsis. *Cell Mol Life Sci*. (2017) 74:3965–87. doi: 10.1007/s00018-017-2610-1

50. Filadelfi AM, Castrucci AM. Comparative aspects of the pineal/melatonin system of poikilothermic vertebrates. *J Pineal Res*. (1996) 20:175–86. doi: 10.1111/j.1600-079X.1996.tb00256.x

51. Roth JJ, Gern WA, Roth EC, Ralph CL, Jacobson E. Nonpineal melatonin in the alligator (Alligator mississippiensis). *Science*. (1980) 210:548–50. doi: 10.1126/science.7423204

52. Lerner AB, Case JD, Mori W, Wright MR. Melatonin in peripheral nerve. *Nature*. (1959) 183:1821. doi: 10.1038/1831821a0

53. Acuna-Castroviejo D, Escames G, Venegas C, Diaz-Casado ME, Lima-Cabello E, Lopez LC, et al. Extrapineal melatonin: sources, regulation, and potential functions. *Cell Mol Life Sci*. (2014) 71:2997–3025. doi: 10.1007/s00018-014-1579-2

54. Damian E. Extra-pineal gland sources of melatonin. *Endocrinologie*. (1977) 15:65–6. doi: 10.1203/00006450-199404000-00002

55. Hazlerigg D, Blix AS, Stokkan KA. Waiting for the sun: the circannual programme of reindeer is delayed by the recurrence of rhythmical melatonin secretion after the arctic night. *J Exp Biol*. (2017) 220:3869–72. doi: 10.1242/jeb.163741

56. Back K, Tan DX, Reiter RJ. Melatonin biosynthesis in plants: multiple pathways catalyze tryptophan to melatonin in the cytoplasm or chloroplasts. *J Pineal Res*. (2016) 61:426–37. doi: 10.1111/jpi.12364

57. Hardeland R, Poeggeler B. Non-vertebrate melatonin. *J Pineal Res*. (2003) 34:233–41. doi: 10.1034/j.1600-079X.2003.00040.x

58. Poeggeler B, Hardeland R. Detection and quantification of melatonin in a dinoflagellate, gonyaulax polyedra: solutions to the problem of methoxyindole destruction in non-vertebrate material. *J Pineal Res*. (1994) 17:1–10. doi: 10.1111/j.1600-079X.1994.tb00106.x

59. Tan DX, Manchester LC, Liu X, Rosales-Corral SA, Acuna-Castroviejo D, Reiter RJ. Mitochondria and chloroplasts as the original sites of melatonin synthesis: A hypothesis related to melatonin's primary function and evolution in eukaryotes. *J Pineal Res*. (2013) 54:127–38. doi: 10.1111/jpi.12026

60. Zhao D, Yu Y, Shen Y, Liu Q, Zhao Z, Sharma R, et al. Melatonin synthesis and function: evolutionary history in animals and plants. *Front Endocrinol (Lausanne)*. (2019) 10:249. doi: 10.3389/fendo.2019.00249

61. Hardeland R, Balzer I, Poeggeler B, Fuhrberg B, Uria H, Behrmann G, et al. On the primary functions of melatonin in evolution: mediation of photoperiodic signals in a unicell, photooxidation, and scavenging of free radicals. *J Pineal Res*. (1995) 18:104–11. doi: 10.1111/j.1600-079X.1995.tb00147.x

62. Lyons TW, Reinhard CT. Early earth: oxygen for heavy-metal fans. *Nature*. (2009) 461:179–81. doi: 10.1038/461179a

63. Manchester LC, Poeggeler B, Alvares FL, Ogden GB, Reiter RJ. Melatonin immunoreactivity in the photosynthetic prokaryote Rhodospirillum rubrum: implications for an ancient antioxidant system. *Cell Mol Biol Res*. (1995) 41:391–5.

64. Duque P, Vieira CP, Vieira J. Advances in novel animal vitamin C biosynthesis pathways and the role of prokaryote-based inferences to understand their origin. *Genes (Basel)*. (2022) 13(10):1917. doi: 10.3390/genes13101917

65. Degli Esposti M, Mentel M, Martin W, Sousa FL. Oxygen reductases in alphaproteobacterial genomes: physiological evolution from low to high oxygen environments. *Front Microbiol*. (2019) 10:499. doi: 10.3389/fmicb.2019.00499

66. Maugars G, Nourizadeh-Lillabadi R, Weltzien FA. New insights into the evolutionary history of melatonin receptors in vertebrates, with particular focus on teleosts. *Front Endocrinol (Lausanne)*. (2020) 11:538196. doi: 10.3389/fendo.2020.538196

67. Reiter RJ, Tan D-X, Manchester L, Terron MP, Flores LJ, Koppisepi S. Medical implications of melatonin: receptor-mediated and receptor-independent actions. *Adv Med Sci (De Gruyter Open)*. (2007) 52:11–28.

68. Dubbels R, Reiter RJ, Klenke E, Goebel A, Schnakenberg E, Ehlers C, et al. Melatonin in edible plants identified by radioimmunoassay and by high performance liquid chromatography-mass spectrometry. *J Pineal Res*. (1995) 18:28–31. doi: 10.1111/j.1600-079X.1995.tb00136.x

69. Hattori A, Migitaka H, Iigo M, Itoh M, Yamamoto K, Ohtani-Kaneko R, et al. Identification of melatonin in plants and its effects on plasma melatonin levels and binding to melatonin receptors in vertebrates. *Biochem Mol Biol Int*. (1995) 35:627–34.

70. Kolar J, Machackova I, Illnerova H, Prinsen E, Van Dongen W, Van Onckelen H. Melatonin in higher plant determined by radioimmunoassay and liquid chromatography-mass spectrometry. *Biol Rhythm Res*. (1995) 26:406–9. doi: 10.1111/j.1600-079X.2005.00276.x

71. Manchester LC, Tan DX, Reiter RJ, Park W, Monis K, Qi W. High levels of melatonin in the seeds of edible plants: possible function in germ tissue protection. *Life Sci*. (2000) 67:3023–9. doi: 10.1016/s0024-3205(00)00896-1

72. Arnao MB, Cano A, Hernandez-Ruiz J. Phytomelatonin: an unexpected molecule with amazing performances in plants. *J Exp Bot*. (2022) 73:5779–800. doi: 10.1093/jxb/erac009

73. Milev NB, Rhee SG, Reddy AB. Cellular timekeeping: it's redox O'clock. *Cold Spring Harb Perspect Biol*. (2018) 10(5):a027698. doi: 10.1101/cshperspect.a027698

74. Yanar K, Simsek B, Cakatay U. Integration of melatonin related redox homeostasis, aging, and circadian rhythm. *Rejuv Res*. (2019) 22:409–19. doi: 10.1089/rej.2018.2159

75. Antolin I, Obst B, Burkhardt S, Hardeland R. Antioxidative protection in a high-melatonin organism: the dinoflagellate Gonyaulax polyedra is rescued from lethal oxidative stress by strongly elevated, but physiologically possible concentrations of melatonin. *J Pineal Res*. (1997) 23:182–90. doi: 10.1111/j.1600-079x.1997.tb00353.x

76. Livrea MA, Tesoriere L, D'Arpa D, Morreale M. Reaction of melatonin with lipoperoxyl radicals in phospholipid bilayers. *Free Radic Biol Med*. (1997) 23:706–11. doi: 10.1016/S0891-5849(97)00018-X

77. Matuszak Z, Reszka K, Chignell CF. Reaction of melatonin and related indoles with hydroxyl radicals: Epr and spin trapping investigations. *Free Radic Biol Med*. (1997) 23:367–72. doi: 10.1016/s0891-5849(96)00614-4

78. Poeggeler B, Reiter RJ, Tan DX, Chen LD, Manchester LC. Melatonin, hydroxyl radical-mediated oxidative damage, and aging: A hypothesis. *J Pineal Res*. (1993) 14:151–68. doi: 10.1111/j.1600-079X.1993.tb00498.x

79. Stasica P, Ulanski P, Rosiak JM. Melatonin as a hydroxyl radical scavenger. *J Pineal Res*. (1998) 25:65–6. doi: 10.1111/j.1600-079X.1998.tb00387.x

80. Tan D-X, Chen LD, Poeggeler B, Manchester LC, Reiter RJ. Melatonin: A potent, endogenous hydroxyl radical scavenger. *Endocr J*. (1993) 1:57–60

81. Turjanski AG, Rosenstein RE, Estrin DA. Reactions of melatonin and related indoles with free radicals: A computational study. *J Med Chem*. (1998) 41:3684–9. doi: 10.1021/jm980117m

82. Zang LY, Cosma G, Gardner H, Vallyathan V. Scavenging of reactive oxygen species by melatonin. *Biochim Biophys Acta*. (1998) 1425:469–77. doi: 10.1016/s0304-4165(98)00099-3

83. Mahal HS, Sharma HS, Mukherjee T. Antioxidant properties of melatonin: A pulse radiolysis study. *Free Radic Biol Med*. (1999) 26:557–65. doi: 10.1016/s0891-5849(98)00226-3

84. Noda Y, Mori A, Liburdy R, Packer L. Melatonin and its precursors scavenge nitric oxide. *J Pineal Res*. (1999) 27:159–63. doi: 10.1111/j.1600-079X.1999.tb00611.x

85. Zhang H, Squadrito GL, Uppu R, Pryor WA. Reaction of peroxynitrite with melatonin: A mechanistic study. *Chem Res Toxicol*. (1999) 12:526–34. doi: 10.1021/tx980243t

86. Galano A, Medina ME, Tan DX, Reiter RJ. Melatonin and its metabolites as copper chelating agents and their role in inhibiting oxidative stress: A physicochemical analysis. *J Pineal Res*. (2015) 58:107–16. doi: 10.1111/jpi.12196

87. Galano A, Tan DX, Reiter RJ. On the free radical scavenging activities of melatonin's metabolites, Afmk and Amk. *J Pineal Res*. (2013) 54:245–57. doi: 10.1111/jpi.12010

88. Tan DX, Manchester LC, Terron MP, Flores LJ, Reiter RJ. One molecule, many derivatives: A never-ending interaction of melatonin with reactive oxygen and nitrogen species? *J Pineal Res*. (2007) 42:28–42. doi: 10.1111/j.1600-079X.2006.00407.x

89. Cordoba-Moreno MO, Santos GC, Muxel SM, Dos Santos-Silva D, Quiles CL, Sousa KDS, et al. Il-10-induced Stat3/Nf-Kappab crosstalk modulates pineal and extra-pineal melatonin synthesis. *J Pineal Res*. (2024) 76:e12923. doi: 10.1111/jpi.12923

90. Hardeland R. Redox biology of melatonin: discriminating between circadian and noncircadian functions. *Antioxid Redox Signal*. (2022) 37:704–25. doi: 10.1089/ars.2021.0275

91. Venegas C, Garcia JA, Escames G, Ortiz F, Lopez A, Doerrier C, et al. Extrapineal melatonin: analysis of its subcellular distribution and daily fluctuations. *J Pineal Res*. (2012) 52:217–27. doi: 10.1111/j.1600-079X.2011.00931.x

92. Reiter RJ, Rosales-Corral S, Tan DX, Jou MJ, Galano A, Xu B. Melatonin as a mitochondria-targeted antioxidant: one of evolution's best ideas. *Cell Mol Life Sci*. (2017) 74:3863–81. doi: 10.1007/s00018-017-2609-7

93. Suofu Y, Li W, Jean-Alphonse FG, Jia J, Khattar NK, Li J, et al. Dual role of mitochondria in producing melatonin and driving GPCR signaling to block cytochrome C release. *Proc Natl Acad Sci USA*. (2017) 114:E7997–8006. doi: 10.1073/pnas.1705768114

94. Nuszkiewicz J, Wozniak A, Szewczyk-Golec K. Ionizing radiation as a source of oxidative stress-the protective role of melatonin and vitamin D. *Int J Mol Sci*. (2020) 21(16):5804. doi: 10.3390/ijms21165804

95. Melchiorri D, Reiter RJ, Attia AM, Hara M, Burgos A, Nistico G. Potent protective effect of melatonin on in vivo paraquat-induced oxidative damage in rats. *Life Sci*. (1995) 56:83–9. doi: 10.1016/0024-3205(94)00417-Q

96. Haghi-Aminjan H, Farhood B, Rahimifard M, Didari T, Baeeri M, Hassani S, et al. The protective role of melatonin in chemotherapy-induced nephrotoxicity: A systematic review of non-clinical studies. *Expert Opin Drug Metab Toxicol*. (2018) 14:937–50. doi: 10.1080/17425255.2018.1513492

97. Colombage R, Singh MB, Bhalla PL. Melatonin and abiotic stress tolerance in crop plants. *Int J Mol Sci*. (2023) 24(8):7447. doi: 10.3390/ijms24087447

98. Shi H, Wang X, Tan DX, Reiter RJ, Chan Z. Comparative physiological and proteomic analyses reveal the actions of melatonin in the reduction of oxidative stress in Bermuda grass (Cynodon dactylon (L). *Pers) J Pineal Res*. (2015) 59:120–31. doi: 10.1111/jpi.12246

99. Moustafa-Farag M, Elkelish A, Dafea M, Khan M, Arnao MB, Abdelhamid MT, et al. Role of melatonin in plant tolerance to soil stressors: salinity, Ph and heavy metals. *Molecules*. (2020) 25(22):5359. doi: 10.3390/molecules25225359

100. Jan R, Asif S, Asaf S, Lubna, Du XX, Park JR, et al. Melatonin alleviates arsenic (as) toxicity in rice plants *via* modulating antioxidant defense system and secondary metabolites and reducing oxidative stress. *Environ pollut*. (2023) 318:120868. doi: 10.1016/j.envpol.2022.120868

101. Arnao MB, Hernandez-Ruiz J. Melatonin: A new plant hormone and/or a plant master regulator? *Trends Plant Sci*. (2019) 24:38–48. doi: 10.1016/j.tplants.2018.10.010

102. Jou MJ, Peng TI, Hsu LF, Jou SB, Reiter RJ, Yang CM, et al. Visualization of melatonin's multiple mitochondrial levels of protection against mitochondrial Ca(2+)-mediated permeability transition and beyond in rat brain astrocytes. *J Pineal Res*. (2010) 48:20–38. doi: 10.1111/j.1600-079X.2009.00721.x

103. Reiter RJ, Sharma R, Pires de Campos Zuccari DA, de Almeida Chuffa LG, Manucha W, Rodriguez C. Melatonin synthesis in and uptake by mitochondria: implications for diseased cells with dysfunctional mitochondria. *Future Med Chem*. (2021) 13:335–9. doi: 10.4155/fmc-2020-0326

104. Cong L, Liu X, Bai Y, Qin Q, Zhao L, Shi Y, et al. Melatonin alleviates pyroptosis by regulating the Sirt3/Foxo3alpha/Ros axis and interacting with apoptosis in atherosclerosis progression. *Biol Res*. (2023) 56:62. doi: 10.1186/s40659-023-00479-6

105. Pi H, Xu S, Reiter RJ, Guo P, Zhang L, Li Y, et al. Sirt3-Sod2-Mros-dependent autophagy in cadmium-induced hepatotoxicity and salvage by melatonin. *Autophagy*. (2015) 11:1037–51. doi: 10.1080/15548627.2015.1052208

106. Lee S, Jeon YM, Jo M, Kim HJ. Overexpression of Sirt3 suppresses oxidative stress-induced neurotoxicity and mitochondrial dysfunction in dopaminergic neuronal cells. *Exp Neurobiol*. (2021) 30:341–55. doi: 10.5607/en21021

107. Zhao Y, Wang Q, Gu D, Huang F, Liu J, Yu L, et al. Melatonin, a phytohormone for enhancing the accumulation of high-value metabolites and stress tolerance in microalgae: applications, mechanisms, and challenges. *Bioresour Technol*. (2024) 393:130093. doi: 10.1016/j.biortech.2023.130093

108. Jockers R, Delagrange P, Dubocovich ML, Markus RP, Renault N, Tosini G, et al. Update on melatonin receptors: Iuphar review 20. *Br J Pharmacol*. (2016) 173:2702–25. doi: 10.1111/bph.13536

109. Klein DC. The pineal gland: A model of neuroendocrine regulation. *Res Publ*. (1978) 56:303–27

110. Klein DC. Photoneural regulation of the mammalian pineal gland. *Ciba Found Symp*. (1985) 117:38–56. doi: 10.1002/9780470720981.ch4

111. Zimmerman S, Reiter RJ. Melatonin and the optics of the human body. *Melatonin Res*. (2019) 2:138–60. doi: 10.32794/mr11250016

112. Slominski A, Tobin DJ, Zmijewski MA, Wortsman J, Paus R. Melatonin in the skin: synthesis, metabolism and functions. *Trends Endocrinol Metab*. (2008) 19:17–24. doi: 10.1016/j.tem.2007.10.007

113. Slominski AT, Kleszczynski K, Semak I, Janjetovic Z, Zmijewski MA, Kim TK, et al. Local melatoninergic system as the protector of skin integrity. *Int J Mol Sci*. (2014) 15:17705–32. doi: 10.3390/ijms151017705

114. Holtkamp CE, Warmus D, Bonowicz K, Gagat M, Linowiecka K, Wolnicka-Glubisz A, et al. Ultraviolet radiation-induced mitochondrial disturbances are attenuated by metabolites of melatonin in human epidermal keratinocytes. *Metabolites*. (2023) 13(7):861. doi: 10.3390/metabo13070861

115. Bocheva G, Slominski RM, Janjetovic Z, Kim TK, Bohm M, Steinbrink K, et al. Protective role of melatonin and its metabolites in skin aging. *Int J Mol Sci*. (2022) 23 (3):1238. doi: 10.3390/ijms23031238

116. Tan DX, Reiter RJ, Zimmerman S, Hardeland R. Melatonin: both a messenger of darkness and a participant in the cellular actions of non-visible solar radiation of near infrared light. *Biol (Basel)*. (2023) 12(1):89. doi: 10.3390/biology12010089

117. Lipko NB. Photobiomodulation: evolution and adaptation. *Photobiomodul Photomed Laser Surg*. (2022) 40:213–33. doi: 10.1089/photob.2021.0145

118. Tan DX, Reiter RJ, Manchester LC, Yan MT, El-Sawi M, Sainz RM, et al. Chemical and physical properties and potential mechanisms: melatonin as a broad spectrum antioxidant and free radical scavenger. *Curr Top Med Chem*. (2002) 2:181–97. doi: 10.2174/1568026023394443

119. Byeon Y, Tan DX, Reiter RJ, Back K. Predominance of 2-hydroxymelatonin over melatonin in plants. *J Pineal Res*. (2015) 59:448–54. doi: 10.1111/jpi.12274

120. Tian X, Kang X, Yan F, Feng L, Huo X, Zhang H, et al. Ros-dependent catalytic mechanism of melatonin metabolism and its application in the measurement of reactive oxygen. *Front Chem*. (2023) 11:1229199. doi: 10.3389/fchem.2023.1229199

121. Samanta S, Seth CS, Roychoudhury A. The molecular paradigm of reactive oxygen species (Ros) and reactive nitrogen species (Rns) with different phytohormone signaling pathways during drought stress in plants. *Plant Physiol Biochem*. (2024) 206:108259. doi: 10.1016/j.plaphy.2023.108259

122. He C, Wang J, Zhang Z, Yang M, Li Y, Tian X, et al. Mitochondria synthesize melatonin to ameliorate its function and improve mice oocyte's quality under in vitro conditions. *Int J Mol Sci*. (2016) 17(6):939. doi: 10.3390/ijms17060939

123. Sakaguchi K, Itoh MT, Takahashi N, Tarumi W, Ishizuka B. The rat oocyte synthesises melatonin. *Reprod Fertil Dev*. (2013) 25:674–82. doi: 10.1071/RD12091

124. Semak I, Naumova M, Korik E, Terekhovich V, Wortsman J, Slominski A. A novel metabolic pathway of melatonin: oxidation by cytochrome C. *Biochemistry*. (2005) 44:9300–7. doi: 10.1021/bi050202d

125. Slominski AT, Zmijewski MA, Semak I, Kim TK, Janjetovic Z, Slominski RM, et al. Melatonin, mitochondria, and the skin. *Cell Mol Life Sci*. (2017) 74:3913–25. doi: 10.1007/s00018-017-2617-7

126. Kerenyi NA, Somogyi E, Sotonyi P. Electron microscopic studies on the aryl-sulphatase activity of the pineal gland. *Acta Histochem*. (1975) 53:192–7.

127. Markus RP, Cecon E, Pires-Lapa MA. Immune-pineal axis: nuclear factor Kappab (Nf-Kb) mediates the shift in the melatonin source from pinealocytes to immune competent cells. *Int J Mol Sci*. (2013) 14:10979–97. doi: 10.3390/ijms140610979

128. Markus RP, Fernandes PA, Kinker GS, da Silveira Cruz-MaChado S, Marcola M. Immune-pineal axis - acute inflammatory responses coordinate melatonin synthesis by pinealocytes and phagocytes. *Br J Pharmacol*. (2018) 175:3239–50. doi: 10.1111/bph.14083

129. Carvalho-Sousa CE, Pereira EP, Kinker GS, Veras M, Ferreira ZS, Barbosa-Nunes FP, et al. Immune-pineal axis protects rat lungs exposed to polluted air. *J Pineal Res*. (2020) 68:e12636. doi: 10.1111/jpi.12636

130. Yawoot N, Govitrapong P, Tocharus C, Tocharus J. Ischemic stroke, obesity, and the anti-inflammatory role of melatonin. *Biofactors*. (2021) 47:41–58. doi: 10.1002/biof.1690

131. Bonmati-Carrion MA, Rol MA. Melatonin as a mediator of the gut microbiota-host interaction: implications for health and disease. *Antioxid (Basel)*. (2023) 13(1):34. doi: 10.3390/antiox13010034

132. Meng X, Li Y, Li S, Zhou Y, Gan RY, Xu DP, et al. Dietary sources and bioactivities of melatonin. *Nutrients*. (2017) 9(4):367. doi: 10.3390/nu9040367

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The proliferation of derivative and redundant studies in endocrinology due to the application of Mendelian Randomisation and other methods to open databases

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One of the challenges for all scientists and clinicians is staying abreast of the increasingly vast literature which advances medical science and influences clinical practice. Publications in the life sciences are growing at the rate of 5.1% per year with a doubling time of 14 years (1). Whilst on the one hand, this represents advances in the life sciences, within this increase there has been a profusion of studies that are based on data, observations, and/or concepts that have not been generated by the authors but are derived from publicly available sources. These have recently been growing at a much faster rate. In comparison to the general growth rate in published life sciences manuscripts of 5.1% per year, according to the publications listed on PubMed over the last decade the average yearly increase in meta-analyses was 26.3%, in bibliometric analyses was 35.2% and Mendelian Randomisation analyses was 147%.

Reviews, meta-analyses and bibliometric studies are all mechanisms to synthesise, coalesce or integrate evidence. Putting pieces of a jigsaw together to glimpse the bigger picture. With the deluge of individual publications across the literature such syntheses of the evidence regarding a specific topic can be extremely useful. Within endocrinology a

quality review aims to clarify how a hormone may be produced or operate in physiology or a specific pathology. Reviews will often take evidence from experimental models, both *in vitro* and animal models, together with human clinical studies to integrate these to consolidate a paradigm for hormone action. These are of value if significantly more evidence has appeared since the last synthesis or if some distinct insight is applied to put the pieces together in a slightly different way to create a different picture, get across a new concept or create a new hypothesis that may stimulate further work to acquire more pieces of the jigsaw. However, if the same pieces are collected together to produce the same picture then this will be of little interest to those in the field who will already know this information and no value to those from outside the field who wish to learn as the information is already available. Many bibliometric studies lack any insightful evaluation of the evidence covered or how it is synthesised. More concerning are syntheses that lack rigour and promote false concepts and hypotheses. This can occur if the selection of reports and/or of data that are included in a synthesis is biased or does not adequately account for the quality aspect of the findings included. There are many other sources of bias that can occur, such as the bias for just publishing positive and significant findings.

Unfortunately, many published syntheses are flawed. Evaluations of systematic reviews and meta-analyses published across biomedical research revealed many to be poorly conducted and conclusions drawn either redundant or misleading (2, 3). The redundancy in some fields can be considerable with up to 20 meta-analyses of the same topic (2). The increase in published meta-analyses, particularly those emanating from China, was reported previously in 2013 (4). In 2013, PubMed listed 13,239 published meta-analyses; by 2023 there were 34,905 listed, indicating that this trend for generating meta-analyses continues upwards. At Frontiers in Endocrinology we received 341 submissions of meta-analyses last year, up from 73 in 2020, with 71.3% of these coming from China. Last year our journal also received 190 submissions describing bibliometric studies, up from 3 in 2020, prompting a clear instruction to authors that such studies will no longer be considered. More open science has provided increasing opportunities for secondary data use, especially in the case of larger data collections that are representative of the wider population, with detailed meta data facilitating use by external research groups. This has led to a proliferation of studies based on datasets such as the National Health and Nutrition Examination Survey (NHANES) (https://wwwn.cdc.gov/nchs/nhanes/analyticguidelines.aspx), meta-analyses combining analyses across several studies, and Mendelian Randomisation (MR) studies (see below)'.

In fact, the most striking increase in derivative studies that we have witnessed is in MR studies; last year Frontiers in Endocrinology received 552 such submissions, up from 4 in 2020. For some of the clinical specialties in our journal this represented over 15% of their total submissions. In 2023, the vast majority of MR submissions emanated from institutions in China (82.3%). The 2023 MR submission rate represented a 21.5-fold increase since 2020. Over the last 15 years there has been a general rapid rise in published MR studies: the total MR publications listed on PubMed in 2010 was just 61; by 2020 this had increased to 899; in 2023 there were 2,968 published. This represents a 48.7-fold increase in MR publications since 2010; over the same time period there was a 4.8-fold increase in bibliometric publications and a 5-fold increase in published meta-analyses. Together this has created a huge additional burden for editors and reviewers of journals, without necessarily delivering to the demands and needs of journal readers who expect the highest standards to be upheld.

MR is based on Mendel's second law of segregation, namely that genetic variants are randomly and independently acquired at gamete formation from the parents. With this method, a genetic variant is used as a susceptibility marker for an exposure [i.e. an instrumental variable (IV)], which is then used to examine whether the exposure is causally related to an outcome, avoiding confounding, bias and reverse causality associated with observational studies, even if the exposure-outcome relationship is itself confounded (5). This can potentially prevent unwarranted further investigations based on incorrect assumptions of causality following observational studies. Initial application of MR used one-sample analyses, based on individual level data usually from a single cohort, where data was available for both the exposure and outcome combined with genetic information.

Two-sample MR analyses was subsequently developed in which gene-exposure data from one source is related to gene-outcome data from a separate source. This enabled MR to be implemented by co-analysing entirely independent genome-wide association studies (GWAS) of different traits, many of which are publicly available. Together with widely available platforms and software for undertaking MR analyses, two-sample MR can be undertaken relatively easily, without deeper understanding and expertise in genetic epidemiology. However, despite the ease with which results can be obtained, performing a rigorous MR study requires careful consideration, planning and interpretation in order to overcome assumptions and pitfalls inherent in this method. Unfortunately, this is not always adhered to, and there has been a profusion of MR publications of varying quality (6–9). That many MR papers have been published without rigorous evaluation of the underlying assumptions means that many invalid 'causal' associations are now in the literature; this has the potential danger of initiating inappropriate research to investigate associations identified as 'causal' by poorly conducted and invalid MR studies (10).

One of the most important assumptions with MR is the no pleiotropy assumption, which assumes that any relationship between the exposure IV and the outcome is mediated solely by the exposure as opposed to some other, pleiotropic pathway. MR Egger and other sensitivity methods have been developed for addressing pleiotropy, and are often included in MR papers. However, though readily automated, these are often under-powered, and other methods may need to be considered. For example, since IVs for bone mineral density (BMD) identified by GWAS are also related to BMI, multivariable MR is required to evaluate causal effects of BMD on osteoarthritis risk independently of the effects of BMI (11). Correlated pleiotropy is a particular form of pleiotropy which arises where two traits, call X and Y, are correlated, for example as a consequence of shared underlying biology (12). Whereas initial MR analysis may indicate a causal relationship of X on Y, if X and Y are correlated, an equivalent relationship is likely to be seen for Y on X. To confirm true causality, bi-directional MR analyses are required to confirm a causal relationship exists in one direction only. Power considerations are

another major limitation in MR analyses. For example, if the IV used in MR analyses is only related to the exposure very weakly, this can lead to weak instrument bias (12). A related issue is that if an MR is applied to examine causal relationships with a range of outcomes, evidence thresholds need to be adjusted accordingly. Further problems can arise due to issues with measurement of the outcome; for example, incomplete phenotype information, time-variations in the exposure, measurement error, survival bias and gene-environment interactions.

As well as methodological issues, a further limitation of many MR studies is the lack of any clear justification. The relevance assumption presumes that there is good biological rationale for investigating the relationship and that the variant is strongly associated with the exposure for which it is employed as a marker. MR studies should only be performed where genuine doubt exists over the causal nature of a relationship between two variables. For example, circulating levels of vitamin D are known to be positively related to a number of health outcomes, such as BMD and fracture risk (13), which could reflect confounding given vitamin D levels are related to sun exposure and hence physical activity. MR analyses to explore these relationships are well justified, in this case finding no evidence of a causal relationship between circulating vitamin D levels and either BMD or fracture risk (12). In contrast, there is no debate as to whether premature menopause causes osteoporosis, and an MR analysis is not justifiable to establish whether a causal relationship exists in this context.

In order to address the poor quality of many published MR studies there have been several published guidelines, with helpful guides on how to conduct a MR study (14, 15), how to evaluate the instrumental variable assumptions (16, 17), how to address bias and quality (18), how to report an MR study with the STROBE-MR framework (Strengthening the Reporting of Observational Studies in Epidemiology Using MR) (19, 20), how to assess the plausibility of an MR (21) and even how to read an MR study (22). The multitude of published guidelines is an indication that undertaking an MR study that is plausible and valid is not as straightforward as may initially seem. Frontiers in Endocrinology adheres strictly to the STROBE-MR guidelines as indicated in our instructions to authors. Despite these numerous published guidelines, we are still witnessing many poor submissions and have to reject the vast majority of submitted MR studies. Other endocrine journals have had similar recent experience (23). The problems of low quality and redundant studies are not restricted to MR studies; the availability of huge databases (such as NHANES and UK Biobank) has led to many other poorly conducted epidemiology studies that offer no insight.

Initially, as few MR studies were submitted, and some editors were unfamiliar with such analyses, the rejections of the submissions to our journal were similar to that for our overall submissions. However, with the recent huge increase in MR submissions, and as editors and reviewers have become familiar with their limitations, the rejection rate has been steadily rising, now over 80% and continuing to increase. The processing of hundreds of such poor-quality submissions adds considerably to the workload of editors and reviewers. To limit overload, we now reject submissions of MR studies that are not accompanied by a completed STROBE-MR checklist. We will similarly be imposing a condition that systematic reviews adhere to the PRISMA guidelines and include a completed checklist with submission and have a conclusion related to endocrinology. In addition, reviews including meta-analyses will have to adhere to the PRISMA extension guidelines and include a completed checklist with submission.

The profusion of mostly derivative studies, and in particular the large number of redundant manuscripts, poses a challenge to the purest ethos of open-access publishing: that it should be open, transparent, inclusive and available. Fundamentally all publications should address a biologically well-defined and reasonable question, relevant to human health and the significant health challenges the world faces, based on a sound hypothesis and that is relevant to the subject. All manuscripts should advance the knowledge-base, revealing new data or new concepts that will drive research and improve clinical practice. With the sharp increase in derivative and redundant manuscripts, together with the advent of manuscripts generated by Artificial Intelligence and 'paper-mills', the challenge will be to minimise the publication of redundant and meaningless manuscripts to ensure that the valuable science is not swamped. As the most cited journal with broad coverage across endocrinology and metabolism and with a commitment to open science, Frontiers in Endocrinology welcomes all credible and insightful submissions. We are, however, constantly revising procedures to filter out redundant manuscripts. With ever increasing numbers of journals open to submissions it requires editors from all journals to be diligent to prevent dilution or distortion of the endocrine literature. The drive to openness and inclusivity must not lose sight of the requirement for value, rigor, quality and expansion of true knowledge.

Author contributions

JT: Writing – original draft, Writing – review & editing. KS: Writing – review & editing. RI: Writing – review & editing. TD: Writing – review & editing. ÅS: Writing – review & editing. ID: Writing – review & editing. JH: Writing – original draft, Writing – review & editing.

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Conflict of interest

ID is an employee of Frontiers Media SA.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Bornmann L, Haunschild R, Mutz R. Growth rates of modern science: a latent piecewise growth curve approach to model publication numbers from established and new literature databases. *Humanit Soc Sci Commun*. (2021) 8:224. doi: 10.1057/s41599-021-00903-w

2. Ioannidis JP. The mass production of redundant, misleading, and conflicted systematic reviews and meta-analyses. *Milbank Q*. (2016) 94:485–514. doi: 10.1111/1468–0009.12210

3. Page MJ, Shamseer L, Altman DG, Tetzlaff J, Sampson M, Tricco AC, et al. Epidemiology and reporting characteristics of systematic reviews of biomedical research: A cross-sectional study. *PloS Med*. (2016) 13:e1002028. doi: 10.1371/journal.pmed.1002028

4. Ioannidis JPA, Chang CQ, Lam TK, Schully SD, Khoury MJ. The geometric increase in meta-analyses from China in the genomic era. *PloS One*. (2013) 8:e65602. doi: 10.1371/journal.pone.0065602

5. Katan MB. Apolipoprotein E isoforms, serum cholesterol, and cancer. *Lancet*. (1986) 1:507–8. doi: 10.1016/s0140–6736(86)92972–7

6. Boef AG, Dekkers OM, Le Cessie S. Mendelian randomization studies: a review of the approaches used and the quality of reporting. *Int J Epidemiol*. (2015) 44:496–511. doi: 10.1093/ije/dyv071

7. Lor GCY, Risch HA, Fung WT, Au Yeung SL, Wong IOL, Zheng W, et al. Cancer Reporting and guidelines for mendelian randomization analysis: A systematic review of oncological studies. *Epidemiol*. (2019) 62:101577. doi: 10.1016/j.canep.2019.101577

8. Islam SN, Ahammed T, Anjum A, Albalawi O, Uddin MJ. Reporting methodological issues of the mendelian randomization studies in health and medical research: a systematic review. *BMC Med Res Methodol*. (2022) 22:21. doi: 10.1186/s12874–022-01504–0

9. Gibson MJ, Spiga F, Campbell A, Khouja JN, Richmond RC, Munafò MR. Reporting and methodological quality of studies that use Mendelian randomisation in UK Biobank: a meta-epidemiological study. *BMJ Evid Based Med*. (2023) 28:103–10. doi: 10.1136/bmjebm-2022–112006

10. Adam D. The causation detector. *Nature*. (2019) 576:196–9. doi: 10.1038/d41586-019-03754-3

11. Hartley A, Sanderson E, Granell R, Paternoster L, Zheng J, Smith GD, et al. Genetics of Osteoarthritis Consortium; Gregson CL, Tobias JH. Using multivariable Mendelian randomization to estimate the causal effect of bone mineral density on osteoarthritis risk, independently of body mass index. *Int J Epidemiol*. (2022) 51:1254–67. doi: 10.1093/ije/dyab251

12. Zheng J, Frysz M, Kemp JP, Evans DM, Davey Smith G, Tobias JH. Use of Mendelian randomization to examine causal inference in osteoporosis. *Front Endocrinol*. (2019) 10:807. doi: 10.3389/fendo.2019.00807

13. Hill TR, Aspray TJ. The role of vitamin D in maintaining bone health in older people. *Ther Adv Musculoskelet Dis*. (2017) 9:89–95. doi: 10.1177/1759720X17692502

14. Haycock PC, Burgess S, Wade KH, Bowden J, Relton C, Davey Smith G. Best (but oft-forgotten) practices: the design, analysis, and interpretation of Mendelian randomization studies. *Am J Clin Nutr*. (2016) 103:965–78. doi: 10.3945/ajcn.115.118216

15. Burgess S, Smith GD, Davies NM, Dudbridge F, Gill D, Glymour MM, et al. Guidelines for performing Mendelian randomization investigations: update for summer 2023. *Wellcome Open Res*. (2019) 4:186. doi: 10.12688/wellcomeopenres.15555.3

16. Glymour MM, Tchetgen Tchetgen EJ, Robins JM. Credible Mendelian randomization studies: approaches for evaluating the instrumental variable assumptions. *Am J Epidemiol*. (2012) 175:332–9. doi: 10.1093/aje/kwr323

17. Burgess S, Small DS, Thompson SG. A review of instrumental variable estimators for Mendelian randomization. *Stat Methods Med Res*. (2017) 5:2333–55. doi: 10.1177/0962280215597579

18. Spiga F, Gibson M, Dawson S, Tilling K, Davey Smith G, Munafò MR, et al. Tools for assessing quality and risk of bias in Mendelian randomization studies: a systematic review. *Int J Epidemiol*. (2023) 52:227–49. doi: 10.1093/ije/dyac149

19. Skrivankova VW, Richmond RC, Woolf BAR, Davies NM, Swanson SA, VanderWeele TJ, et al. Strengthening the reporting of observational studies in epidemiology using mendelian randomisation (STROBE-MR): explanation and elaboration. *BMJ*. (2021) 375:n2233. doi: 10.1136/bmj.n2233

20. Skrivankova VW, Richmond RC, Woolf BAR, Yarmolinsky J, Davies NM, Swanson SA, et al. Strengthening the reporting of observational studies in epidemiology using mendelian randomization: the STROBE-MR statement. *JAMA*. (2021) 326:1614–21. doi: 10.1001/jama.2021.18236

21. Burgess S, Butterworth AS, Thompson JR. Beyond Mendelian randomization: how to interpret evidence of shared genetic predictors. *J Clin Epidemiol*. (2016) 69:208–16. doi: 10.1016/j.jclinepi.2015.08.001

22. Davies NM, Holmes MV, Smith GD. Reading Mendelian randomisation studies: a guide, glossary, and checklist for clinicians. *BMJ*. (2018) 362:k601. doi: 10.1136/bmj.k601

23. Kjaergaard AD, Smith GD, Stewart P. Mendelian randomization studies in endocrinology: raising the quality bar for submissions and publications in the journal of clinical endocrinology & Metabolism. *J Clin Endocrinol Metab*. (2023) 109:1–3. doi: 10.1210/clinem/dgad569

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Circulating miR-28-5p is overexpressed in patients with sarcopenia despite long-term remission of Cushing's syndrome: a pilot study

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Introduction: Patients with Cushing's syndrome (CS) in remission show sustained fatigue, myopathy, and an increased prevalence of sarcopenia. The mechanisms that determine these persistent muscle problems are not well known. We aimed to identify circulating microRNAs (miRNAs) with differential expression that could be potential biomarkers for the diagnosis and/or prognosis in CS.

Patients and methods: Thirty-six women in sustained remission for 13 ± 7 years (mean ± SD) from CS, with a median age (IQ range) of 51 (45.2–60) years and mean ± SD BMI of 27 ± 4 Kg/m^2, and 36 matched healthy controls were investigated. In 7 patients sarcopenia was present according to the European Working Group on Sarcopenia in Older People (EWGSOP) criteria. Small RNA libraries were generated and indexed using a modified Illumina TruSeq small RNA-sequencing protocol. MiRNAs were identified in plasma using bioinformatic analysis, and validation was carried out using RT-qPCR. For the validation, Taqman probes were performed on QuantStudio 5 equipment (Applied Biosystems).

Results: In a first discovery group using RNA-sequencing, plasma samples of 18 CS patients and 18 healthy subjects were investigated; circulating miR-28-5p, miR-495-3p and miR-654-5p were upregulated in CS patients as compared with controls ($p<0.05$). In a validation study of the 3 upregulated miRNAs in 36 patients and 26 controls, no differences were observed by RT-qPCR; however, the

expression of circulating miR-28-5p was upregulated in CS patients with sarcopenia as compared with those without (AUC for fold-change in the ROC analysis, 0.798; p=0.0156). The optimized cut-off value for miR-28-5p to identify CS patients with sarcopenia was 3.80, which yielded a sensitivity of 86% and a specificity of 69%.

Conclusion: MiR-28-5p, a muscle-specific microRNA involved in myotube proliferation and differentiation *in vivo*, may serve as an independent non-invasive biomarker for identifying CS patients at high-risk of sarcopenia despite biochemical remission.

KEYWORDS

microRNA, miR-28-5p, myomiRs, sarcopenia, myopathy, Cushing syndrome

Introduction

Cushing's syndrome (CS) develops because of endogenous hypercortisolism of pituitary or adrenal origin, and more rarely due to ectopic secretion of ACTH. It is a rare disease, which is treated by surgical excision of the originating lesion; drug therapy is also available if surgery is contraindicated, or unsuccessful, and pituitary irradiation is a possibility in recurrent or aggressive disease (1, 2). Over two thirds of patients treated for CS complain of muscle weakness, mainly affecting the pelvic girdle and lower limbs (1–3). In active disease, skeletal muscle mass is decreased in comparison to body mass index (BMI)-matched controls (1, 4–6). Furthermore, it is known that steroids inhibit protein synthesis and enhance protein catabolism, with a consequent selective depletion of contractile proteins, myofibrillar degradation and atrophy of type IIa, fast-twitch muscle fibers (4). Patients' perception of muscle weakness despite endocrine "cure" is confirmed by poorer muscle performance despite unchanged muscle mass in women in remission, as compared with age-, and BMI-matched female controls (7). This muscle dysfunction is associated with increased intramuscular fatty infiltration and changes in muscle composition and architecture, which persist long term after remission of hypercortisolemia (7, 8). On the other hand, sarcopenia is currently considered by the European Working Group on Sarcopenia in Older People 2 -EWGSOP2- a muscle disease characterized by reduced muscle strength and function, but not necessarily associated with low muscle mass (9). We have previously shown that sarcopenia is increased in "cured" CS patients compared to matched controls and impacts considerably on patients perceived health related quality of life (10).

The mechanisms that determine muscle dysfunction after chronic exposure to hypercortisolemia are not well understood. In recent years, ample evidence has been published on the prominent role of microRNAs (miRNAs) in regulating skeletal muscle plasticity and functionality as well as fiber-type specificity and metabolic properties (11). MiRNAs are small non-coding RNA molecules involved in the post-transcriptional regulation of gene expression by binding to their target messenger RNAs (mRNAs). Some miRNAs are ubiquitously expressed in tissue, while others are tissue-specific or tissue enriched; miRNAs can also be expressed in biological fluids such as serum, saliva or urine, where they are resistant to degradation by RNAases (12). Skeletal muscle-specific miRNAs ("myomiRNAs") are involved in myoblast proliferation, differentiation and regeneration, and changes in their circulating levels have been described in several conditions associated with abnormal muscle organization and function, including specific muscle diseases and age-related sarcopenia and frailty (13–16). Moreover, myomiRNAs have been suggested as new biomarkers of physiological and pathological muscle processes (14, 17–22). Furthermore, in a C2C12 mouse myocyte model, hydrocortisone induced overexpression of miR-133a-3p and atrophic signals, also observed in patients with active CS, leading the authors to suggest that this myomiRNA might be a potential biomarker to discriminate between healthy controls and patients with CS (23). Given that prior exposure to hypercortisolism in the context of CS determines residual musculoskeletal morbidity, we aimed at evaluating differentially expressed miRNAs in plasma of patients with CS in remission, and whether they are related to sarcopenia.

Patients and methods

Patients

Thirty-six women with CS in remission and 36 age- and BMI-matched female controls were included. Diagnosis of CS was reached after evaluation of clinical, biochemical, and radiological data, based on international guidelines (24) as previously reported (7). Seven patients had sarcopenia according to the definition of the European Working Group on Sarcopenia in Older People (EWGSOP), as previously described (10). Inclusion criteria were age up to 65 years and in remission a minimum of 3 years. Twenty-eight patients had

Cushing's disease (CD) due to a pituitary microadenoma (n = 25) or a macroadenoma (n = 3) and 8 had an adrenal adenoma. The median (interquartile range) duration of hypercortisolism (defined as the time since initial symptoms, as referred by patients, until final diagnosis of CS) was 33 (12–36) months.

In the 28 CD patients transsphenoidal surgery was performed a median of 154 (117) months before the study; 30 patients (83%) received preoperative treatment with steroidogenesis inhibitors to control clinical symptoms of hypercortisolism; 7 (19%) had undergone radiotherapy a median of 142 (72–180) months after unsuccessful surgery (n = 1) or relapse (n = 6). Adrenalectomy was performed in the 8 patients with an adrenal adenoma a median of 100 months (89–38) months before the study. Mean (± SD) time of remission (time since diagnostic confirmation of remission to study entry) was 13 ± 7 years (median, 13 [6–14] and range, 1–17 years]. Remission was considered if adrenal insufficiency (basal morning cortisol <171 nmol/L [<6.2 µg/dL] and/or undetectable 24-h free urinary cortisol) was observed, or morning cortisol suppressed <50 nmol/L (<1.8 µg/dL) after an overnight 1 mg dexamethasone suppression test. Hydrocortisone (HC) replacement (10 to 20 mg/day) was required for a median of 19 (0–24) months after surgery in 30 patients (83%); median time free from HC replacement was 84 (36–140) months. At study entry, 3 patients were still on HC substitution therapy at a stable dose of 20 mg/day (mean ± SD duration of treatment 148 ± 35 months), 3 had growth hormone deficiency (2 treated with recombinant human GH for a mean ± SD duration of 38 ± 42 months); 5 were hypothyroid (3 due to TSH deficiency and 2 due to primary hypothyroidism), on stable replacement doses (65 ± 22 µg/day) of L-thyroxine (mean ± SD duration of treatment 127 ± 32 months). Fifty-eight percent (n=21) were postmenopausal (mean ± SD duration of menopause of 96 ± 61 months). No patient had postoperative gonadotropin deficiency nor was treated with oestrogen/progesterone hormone replacement. Exclusion criteria included being older than 65 years, active disease, inflammatory disorders, diabetes mellitus, kidney, liver or neurological dysfunctions, malignancies, documented physical disability or motility limitations and treatment with local or systemic glucocorticoids during the previous year.

For each of the 36 CS patients, a female blood donor matched for age, BMI, menopausal status and degree of physical activity was identified and recruited after consent to participate (Table 1). Clinical data on disease characteristics and physical examination were collected as well as fasting blood samples in EDTA tubes for RNA analysis.

The study was approved by the ethics committee of our institution (IIB-CEIC), and all subjects gave full, written consent.

RNA extraction and quantification

Blood samples from CS patients and controls were centrifuged at 2500 rpm for 10 minutes to separate the plasma, followed by a second centrifugation at 16000g and 4 °C for 10 minutes. Absorbance at 414nm and 385nm were measured in NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Scientific, DE, USA) to check the quality of plasma and discard those samples that had A414/385 >3, indicative of haemolysis (n=10 controls). Plasma samples were stored at −80°C until RNA extraction. Cell-free total RNA (including miRNAs) was extracted from 400 µL of plasma using the miRNeasy Serum/Plasma kit (Qiagen, CA, USA), following the manufacturer's protocol. The free RNA was eluted with 25 µL of RNAse-free water. The concentration of cell-free total RNA (including miRNAs) was quantified using NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Scientific, DE, USA).

Library preparation and small RNA-sequencing of the discovery group

To perform small RNA libraries, we selected a discovery group of samples from 18 CS patients (6 with sarcopenia) and 18 healthy subjects (Table 1). The libraries were generated and indexed using a modified Illumina TruSeq small RNA protocol. In this modified protocol the libraries were size selected (range 90-170pb) using the Blue Pippin instrument (Sage Science, Beverly, MA, USA). A positive RNA control was included (Thermo Fisher Scientific Human Brain Total RNA catalogue #AM7962). SmallRNA-sequencing (smallRNA-seq) was performed by single-end sequencing on Illumina NextSeq550 platform on High Output 1x50pb RUN (NextSeq 500/550 High Output v2 75 cycles kit, FC-404-2005).

TABLE 1 Age and Body Mass Index (BMI) of the study groups (mean ± SD).

DISCOVERY GROUP	CONTROLS (n=18)	CS (n=18)	CONTROLS Vs. CS	WITHOUT SARCOPENIA (n=12)	WITH SARCOPENIA (n=6)	WITHOUT Vs. WITH SARCOPENIA
Age years	53.1 ± 9.1	56 ± 6	P = 0.28	54.9 ± 6.2	58.2 ± 5.5	P = 0.3
BMI Kg/m^2	27.5 ± 3.8	27.5 ± 3.6	P = 0.94	27.3 ± 3.3	27.3 ± 3.9	P = 0.79
VALIDATION GROUP	**CONTROL (n=26)**	**CS (n=36)**	**CONTROLS Vs. CS**	**WITHOUT SARCOPENIA (n=29)**	**WITH SARCOPENIA (n=7)**	**WITHOUT Vs. WITH SARCOPENIA**
Age years	50.3 ± 12.4	49.9 ± 11.6	P = 0.88	48.4 ± 12	56.1 ± 7.3	P = 0.16
BMI Kg/m^2	27 ± 4.1	26.6 ± 3.7	P = 0.89	26.1 ± 3.7	28.7 ± 3.3	P = 0.1

The discovery group was formed by 18 patients with Cushing's syndrome (CS) of which 6 had sarcopenia, and 18 controls; the validation group included 36 CS patients, of which 7 had sarcopenia, and 26 controls. No significant differences (p-value <0.05) were observed between groups.

Differential expression analysis of the discovery group

The first step was to assess the quality of the Illumina raw sequences and trimming to remove sequencing adapters and low-quality bases. Thereafter, sequences were mapped against the human GRCh38 reference sequence, taken from Ensembl. After that, the intersection between the aligned position of reads and the miRNA coordinates taken from miRBase v22 was performed. The alignment and quantification steps were performed using Subread and Rsubread Packages.

Prediction of miRNA targets and over-representation analysis of the discovery group

Firstly, we accessed miRanda and DIANA-microT-CDS from the DIANA web server (25), to predict messenger RNAs (mRNAs) which are potential targets of miRNAs. Moreover, we used TarBase v7.0 to identify those mRNAs which have been already validated as targets of our miRNA candidates. An Over-Representation Analysis (ORA) was carried out, using Gene Ontology (GO) terms and the Kyoto Encyclopaedia of Genes and Genomes (KEGG), using as default parameters experimentally supported interactions from DIANA TarBase v.7.0 with a p-value threshold of 0.001, and a microT threshold of 0.8. To reduce the number of false positive miRNA targets, we applied a false discovery rate (FDR) correction to the selected KEGG pathways. The algorithm used in this analysis was a one-tailed Fisher's exact test.

Validation by Real-time qPCR of miRNA signatures from plasma in a validation group

To confirm the data from the sequencing experiments new samples from CS and healthy controls were included. Specifically, the 18 samples of CS patients and the 18 of healthy controls of the discovery group were used and a further 18 CS patient samples and 8 healthy controls were added, resulting in a total group of 36 patients with CS and 26 controls.

Reverse transcription reactions were performed using the TaqMan miRNA Reverse Transcription kit and miRNA-specific stem-loop primers (Part No. 4366597, Applied Biosystems. Inc, CA; USA) and 100 ng of input cell-free RNA in a 20 µL RT reaction. Real-time PCR reactions were performed in triplicate, in scaled-down 10 µL reaction volumes using 5 µL TaqMan 2x Universal PCR Master Mix (Applied Biosystems. Inc, CA; USA) with No UNG, 0.5 µL TaqMan Small RNA assay (20x) (Applied Biosystems. Inc, CA; USA), miR-28-5p (000411), miR-495-3p (001663), miR-654-5p (001611)], 3.5 µL of nuclease free water and 1 µL of RT product. Real-time PCR was carried out on an Applied BioSystems QuantStudio 5 (Applied Biosystems. Inc, CA; USA) programmed as follows: 50°C for 2 minutes, 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. We used miR-191-5p (002299), one of the most stable miRNAs in terms of read counts, as previously described as an endogenous control (26–28) to normalize the expression in plasma samples. All the fold-change data were obtained using the delta-delta cycle threshold (Ct) method ($2^{-\Delta\Delta CT}$) (29).

Validation of miRNAs as biomarkers

To assess differences between patients and controls and between patients with and without sarcopenia, Mann-Witney tests to compare miRNA fold-change values as a continuous variable in the 3 different groups were performed (healthy controls, CS with sarcopenia and CS without sarcopenia).

The miRNA diagnostic test utility was validated by a ROC curve analysis, measuring the area under the curve, diagnostic sensitivity and specificity, and positive and negative predictive values. Optimal cut-off points were determined by highest sensitivity plus specificity and efficiency values.

P-values below 0.05 were considered significant. Data analysis was performed using Graphpad Prism 8.0 (GraphPad Software, CA, USA).

Results

Clinical characteristics of Cushing's syndrome patients and controls

The characteristics of the study populations are shown in Table 1. CS patients had a median age (interquartile range) of 51 (45.2–60) years and a mean (± SD) BMI of 27 ± 4 Kg/m2] and did not differ from the healthy controls; both groups did not differ as far as other clinical features (including time since menopause, and duration of remission, since diagnostic confirmation), as previously described (10). For the discovery group, 18 patients with CS and 18 healthy controls were investigated; 6 of the CS patients had sarcopenia. For the validation study there were 36 CS patients of which 7 had sarcopenia and 26 healthy controls.

Identification of differentially expressed miRNAs by small RNA-sequencing

The expression analysis of miRNAs in the 18 patients with CS compared to the 18 healthy controls of the discovery group using small RNA-sequencing showed differential expression of 3 miRNAs, namely miR-28-5p, miR-495-3p, and miR-654-5p (false discovery rate < 0.05). As shown in Figure 1, Supplementary Table 1 all these miRNAs were upregulated in patients compared to controls.

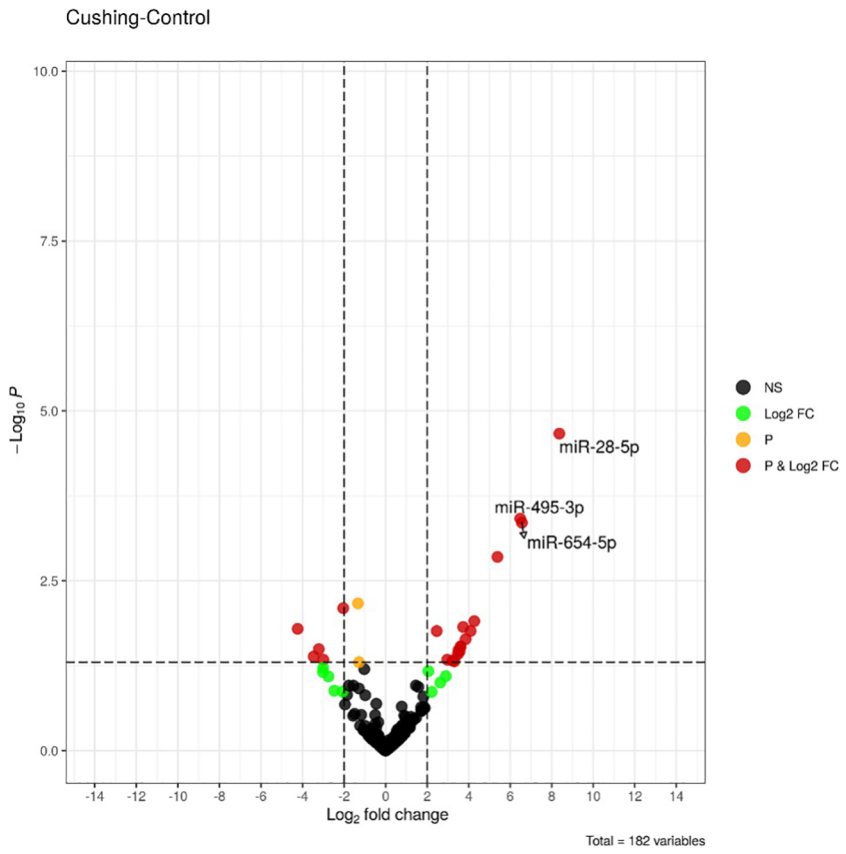

FIGURE 1
Volcano-plot of differentially expressed miRNAs between healthy controls (n = 18) and Cushing's syndrome patients (n = 18) in the discovery group. Vertical lines indicate the threshold for a relative expression fold change (FC) of above 2 or below 2-fold compared to controls. The horizontal line represents the threshold of a 0.05 p-value. BLACK DOTS, NS: miRNAs with no significant change; GREEN DOTS, Log2FC: miRNAs with Log2 fold change > 2 in absolute values; ORANGE DOTS, P: miRNAs with a p-value < 0.05; RED DOTS, P&Log2FC: miRNAs with a p-value < 0.05 and a Log2 fold change > 2 in absolute values. miRNAs with false discovery rate (FDR) less <0.05 are labelled.

Enrichment analysis of miRNA targets and pathways in the context of Cushing's syndrome

To clarify the role of the identified upregulated miRNAs in CS, we analyzed biochemical networks that were regulated by these 3 miRNAs. After ORA analysis, using GO terms and the KEGG pathways significantly regulated by these altered miRNAs, we found a total of 98 GO terms with a p-value of less than 0.05, 81 corresponding to biological processes terms, 11 corresponding to cellular component terms, and 6 corresponding to molecular functions (Supplementary Table 2). The most significant biological processes terms are shown in Figure 2. These include skeletal system development, aging, and muscle cell proliferation. The KEGG analysis, showed 26 pathways with an FDR of less than 0.05 (Supplementary Table 3). The most significant pathways observed, considering the physiopathology of CS, are the MAPK signaling pathway, cellular senescence, and Tumor necrosis factor α (TNFα) signaling pathway (Figure 3).

Validation of the differentially expressed miRNAs by RT-qPCR

The 3 differentially expressed miRNAs detected by smallRNA-seq were validated by RT-qPCR, in 36 CS patients and 26 controls. The relative expression levels for each miRNA were calculated in all the samples analyzed by smallRNA-seq and RT-qPCR, respectively. No differences in plasma miRNAs present in CS patients and controls were observed, in contrast with the results obtained by smallRNA-seq in the discovery phase (Figures 4A–C).

Phenotypic characterization of Cushing's syndrome patients with and without sarcopenia according to miRNA expression

Due to the absence of significant differences observed in the validation assay by RT-qPCR, we evaluated the results in a second approach. Considering the results of the enrichment analysis that

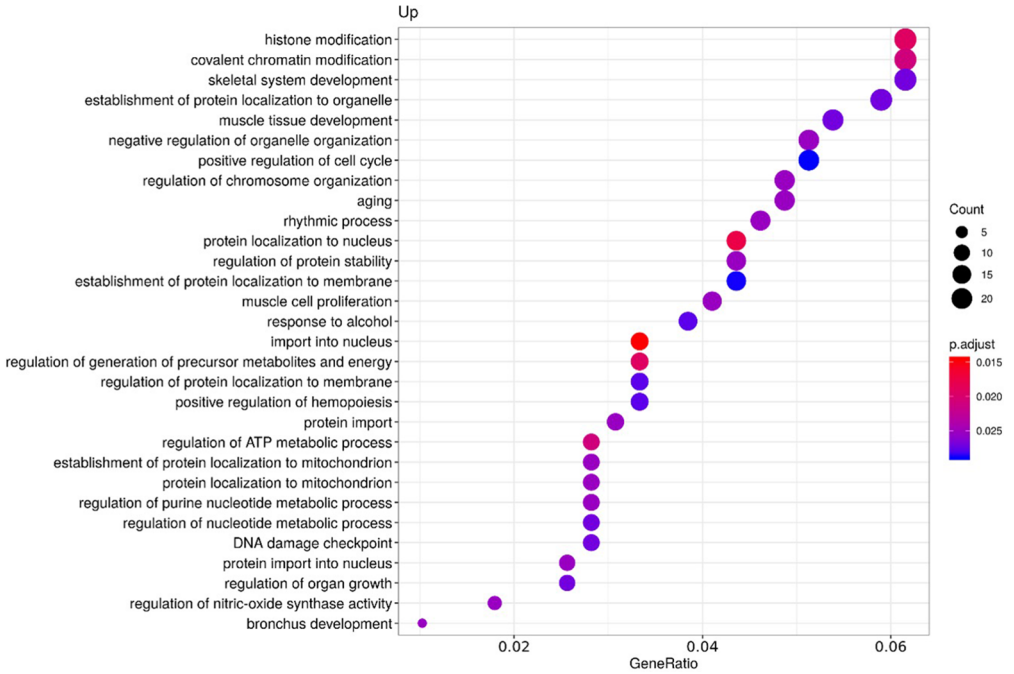

FIGURE 2
Functional enrichment by Over-representation analysis (ORA) using Gene Ontology (GO) terms. GO terms are classified according to the ratio of the number of target genes for each miRNA included in each GO term from the total of genes included in the GO term (Gene ratio), the number of counts per million for each miRNA in each GO term (Count) and the p-value adjusted by the Fisher test (p. adjust).

point out the relevance of these miRNAs in muscle cell proliferation and aging, we analyzed the expression of the 3 upregulated plasma miRNAs in different clinical subgroups, namely 7 CS patients with sarcopenia and 29 without sarcopenia, aimed at identifying specific miRNA signatures. Although no differences in miR-495-3p and miR-654-5p expression were observed, miR-28-5p was upregulated in the 7 CS patients with sarcopenia when compared with those without sarcopenia (Figures 4D–F).

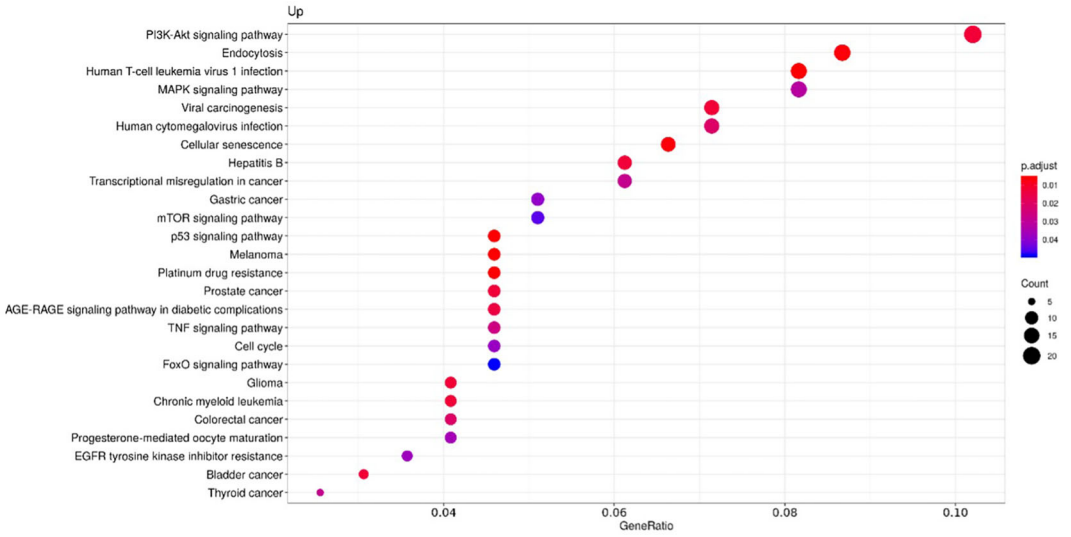

FIGURE 3
Functional enrichment by Over-representation analysis (ORA) using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways. KEGG pathways are classified according to the ratio of the number of target genes for each miRNA included in each pathway from the total of genes of the pathway (Gene ratio), the number of counts per million for each miRNA in each pathway (Count) and the p-value adjusted by the Fisher test (p. adjust).

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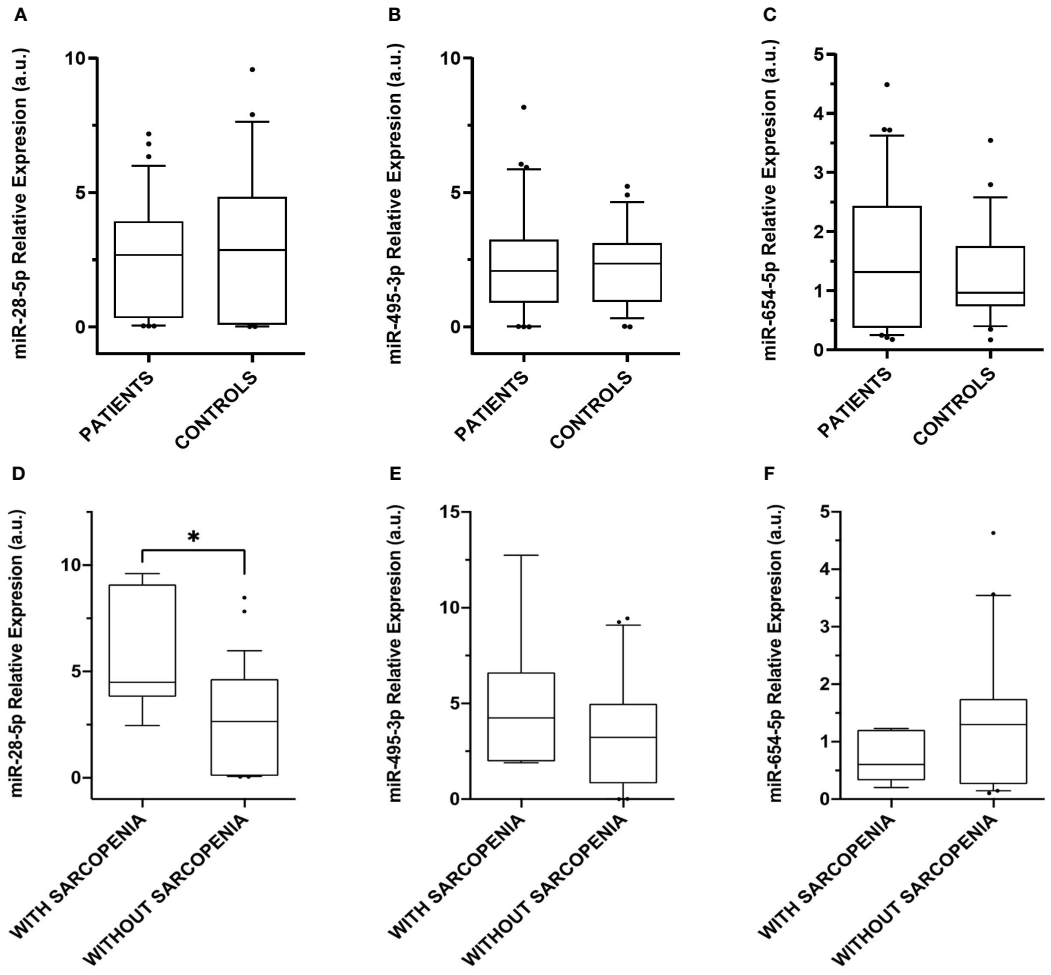

FIGURE 4
Relative expression levels of the miRNAs differentially expressed in plasma. Box plot levels of **(A)** miR-28-5p (p-value = 0.49); **(B)** miR-495-3p (p-value = 0.88), and **(C)** miR-654-5p (p-value = 0.47) in 36 CS patients compared to 26 controls; **(D)** miR-28-5p (* p-value = 0.01); **(E)** miR-495-3p (p-value = 0.43), and **(F)** miR-654-5p (p-value = 0.16) in CS patients without sarcopenia (n = 29) and CS with sarcopenia (n = 7). Expression levels of the miRNAs were normalized to miR-191-5p. The lines inside the boxes denote the medians. The boxes mark the interval between the 25th and 75th percentiles. The whiskers denote the interval between the 10th and 90th percentiles. Filled circles indicate data points outside the 10th and 90th percentiles. Differences were determined using Mann-Whitney tests. P-values were two-tailed and significant if < 0.05.

miR-28-5p as a phenotypic biomarker of sarcopenia in Cushing's syndrome

Given the increase of miR-28-5p in CS patients with sarcopenia, its use as a possible biomarker of sarcopenia was evaluated. A ROC curve identified differences between the miR-28-5p fold change in CS patients with and without sarcopenia, with an AUC of 0.7980 (p=0.0156) and an optimal cut- off value for the fold change of 3.80, with a sensitivity of 86% and a specificity of 69% (Figure 5).

Discussion

In this pilot study, we have found that patients with CS in remission and sarcopenia have higher circulating levels of miR-28-5p, a muscle-specific microRNA involved in myotube proliferation and differentiation, as compared with their counterparts without sarcopenia. A frequent complaint in patients diagnosed with CS is that despite remission, they suffer from sustained fatigue and myopathy, mainly affecting the pelvic girdle and lower limbs (1–3). The mechanisms that determine these persistent muscle problems are not well understood. Our findings suggest that plasma miR-28-5p may be a minimally invasive biomarker to identify those patients with prior CS who maintain a high-risk of sarcopenia despite normalization of cortisol levels and may contribute to discover physiological and molecular pathways underlying this disease.

Although in the first discovery approach using smallRNA-sequencing we identified an overrepresentation of 3 plasma miRNAs (miR-28-5p, miR-495-3p, and miR-654-5p) in CS patients compared to matched healthy control subjects, these results were not confirmed by RT-qPCR in the validation phase, where no differences in the expression of these 3 miRNAs were observed when comparing the whole group of CS patients with controls. The reason for this discrepancy is currently unclear; miRNA expression is known to be highly variable between individuals and is often age-dependent (30). Additionally, myomiRNAs function in a complex fashion in muscle

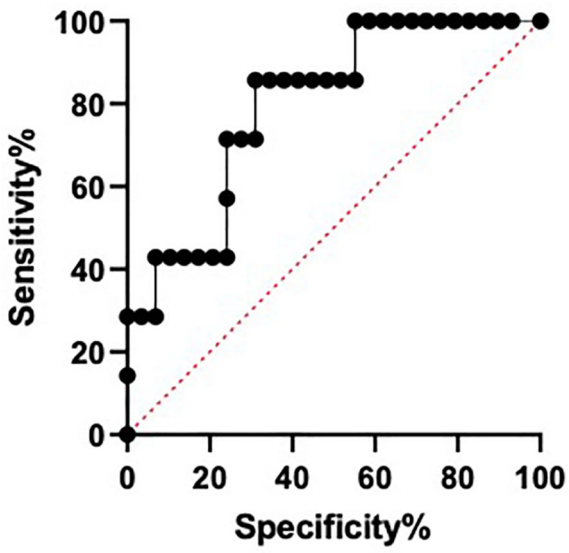

FIGURE 5
Area under the curve of receiver operating characteristic (ROC) for miR-28-5p as a possible biomarker of sarcopenia in Cushing´s syndrome.

tissues, potentially 'buffering' against physiological and pathological changes to maintain homeostasis. They are thought to target multiple gene transcripts in the same pathway to ensure a biological outcome. They may also display cooperativity and redundancy of different miRNAs by synergistically targeting the same transcript (31). Variations in the differential expression of miRNAs may depend on the methodology used (i.e., smallRNAseq or RT-qPCR), given the different normalization approach used to calculate the relative expression of miRNAs for each method. In this regard, small RNA-sequencing uses total counts of reads for each miRNA and the relative expression is calculated by normalizing from the whole counts obtained, while in RT-qPCR relative expression is calculated using an endogenous control to normalize relative expression of CS patients compared to the control subjects. Although it would be interesting to investigate other subgroups of patients with CS, the rarity of this disease prevents the study of large numbers of individuals to further explain the results obtained by RT-qPCR.

Since sequencing did not disclose any differences in the 3 miRNAs analyzed, another approach was adopted, based on the evidence from our enrichment analysis and the literature, by linking these miRNAs to sarcopenia, and evaluating the expression of miRNAs in CS patients with or without sarcopenia. With this approach overexpression of miR-28-5p in serum of patients in long-term remission of CS who suffer from sarcopenia as persistent morbidity was evidenced, compared to CS patients without this muscle dysfunction.

Changes in the expression of several members of the miR-28 family during muscle cell differentiation (32, 33) and in response to stress and exercise (22, 33, 34) suggest that they contribute to the control of muscle cell proliferation, tissue development, muscle regeneration and homeostasis (21, 35–38). Furthermore, the expression of skeletal muscle N-myc downstream-regulated gene 2 (NDRG2) protein is increased during muscle differentiation and development both *in vitro* and *in vivo* (39, 40), promoting myoblast proliferation (31). Overexpression of miR-23a, -23b and -28 in skeletal muscle cells of mice were shown to co-regulate mouse Ndrg2 in the presence of dexamethasone (31). These specific *in vitro* stress conditions, namely in the presence of a synthetic glucocorticoid like dexamethasone, represents a non-physiological condition for the regulation of Ndrg2. However, it could be compared to the chronic exposure to hypercortisolism typical of CS; the fact that in those CS patients with sarcopenia, i.e., with most severe permanent muscle dysfunction, miR-28 was also upregulated, would suggest that both may be linked. Whether miR-28 acts on its own or cooperatively with other miRNAs under similar stress conditions *in vivo*, targeting Ndrg2 is still unclear, as well as if it could represent a potential therapeutic target in catabolic muscle wasting conditions (31).

Further evidence of a relationship between skeletal muscle miR-28 and muscle development has been reported; namely downregulation of miR-28 was observed after aerobic exercise training for 6 weeks in healthy individuals - which will enhance muscle development (41, 42) -, as well as in goat kids, where downregulation of miR-28 promoted myoblast proliferation and differentiation (39). An interesting point for miR-28-5p is its possible implication in aging and myopathy. One target of this miRNA is nuclear factor erythroid 2-related factor 2 (NRF2). Huang et al. demonstrated that a deficiency of Nrf2 in a mouse model was associated with increased frailty and sarcopenia during aging, by altering mitochondrial biogenesis and dynamics (43).

On evaluating the relevant GO terms identified in our enrichment analysis using the KEGG pathways, muscle tissue development and muscle cell proliferation were identified. Both pathways contain common genes regulated by miRNAs, like the *MEF2D* gene, which encodes for the Myocyte-specific enhancer factor 2D that is targeted by miR-28-5p and miR-654-5p; or the *ZBTB18* gene that encodes for Zinc finger and BTB domain-containing protein 18, a transcriptional repressor that regulates myogenesis (44), also targeted by miR-28-5p. These terms are important in the context of CS since muscle wasting and sarcopenia are common complaints in this disease (10, 45). Both miR-654-5p and miR-28-5p have similar effects on vascular smooth muscle cell proliferation and migration, namely an inhibitory effect if they are upregulated and a stimulatory effect when they are downregulated, via the FOXO pathway (46) or by directly targeting ADAMTS-7 (a disintegrin and metalloproteinase with thrombospondin motifs-7) (47). But to the best of our knowledge, no reports on miR-654-5p related to skeletal muscle have been described to date.

Considering the pathophysiology of CS, the MAPK/ERK signaling pathway is a further identified KEGG term; interestingly, it has also been described to be involved in sarcopenia related to aging (32–34). In fact, miR-28-5p targets two genes included in this pathway, namely Insulin-like growth factor-I (IGF-I) and serum response factor (SRF), both related to sarcopenia and that play a role as regulators of muscle atrophy (21, 35–38). Cellular senescence is another relevant pathway identified, since CS has been associated to premature aging and telomere length shortening, as well as with dyslipidemia, osteoporosis, glucose intolerance and elevation of chronic inflammatory markers (48, 49). Additionally, the tumor necrosis factor α (TNFα) signaling pathway was also overrepresented. We have shown that plasma soluble TNFα receptors (sTNF-R1 and sTNF-

R2) are increased in long-term cured CS patients (50) and its overexpression may play a significant role in residual complications like myopathy and muscle atrophy (51). One of the genes of the TNFα signaling pathway is C-C motif chemokine ligand 2 (CCL2) a promotor of myogenesis via the AKT/mTOR signaling pathway, recently associated to active CS (23). They report overexpression of miR-133a-3p in both mouse myocytes exposed *in vitro* to hydrocortisone and serum of patients with active CS (27 patients with CD including 6 males and 10 with adrenal CS, including 2 males), where it was higher than in controls, leading them to suggest that circulating miR-133a-3p could be a promising biomarker of hypercortisolism. In the present study, which differed in the sense that patients were in long-term remission of CS and included both genders, this myomiR was not found to be differentially expressed. Further investigations will be necessary to better understand the role of these myomiRs in CS, both active and in remission.

An attempt to use circulating microRNA expression to identify markers of pituitary CD versus adrenal cortisol-producing adenoma reported upregulation of miR-182-5p using qPCR in the CD group but no differences between controls and the whole CS cohort from the German Cushing's registry (52).

Among the limitations of the present pilot study are its cross-sectional design, which prevents from inferring causality from our data. Additionally, the small groups of patients is practically unavoidable in a rare disease like CS and the limitation of the number of patients may impact directly in the differences observed in the expression of miRNAs detected by smallRNA-seq and RT-qPCR, since their results depend on the different methods used for normalizing the relative expression of miRNAs. Only women were included given the greater prevalence of CS in the female gender (above 70%) which precluded including a sufficient number of men. However, a strength of our study is the detailed screening of sarcopenia in the CS patients with strict criteria, using both physical performance indices and imaging parameters, as defined by the European Working group on Sarcopenia in Older People 2 -EWGSOP2- (9), considered as the gold standard to measure the actual ratio of muscle and fat.

In summary, we have identified free plasma miR-28-5p as a possible non-invasive potential biomarker of the presence of sarcopenia as a long-term persistent consequence of CS, despite attaining remission of hypercortisolemia. Identifying these patients with sarcopenia to offer an adapted exercise program would very likely benefit their long-term prognosis, since improving muscle weakness can prevent falls, as well as benefitting perceived health and quality of life (10, 53). Future studies should confirm whether patients with sarcopenia of another origin (not related to CS) also exhibit this upregulation of miR-28-5p.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below. GEO (Gene Expression Omnibus) repository, accession number GSE271649.

Ethics statement

The studies involving humans were approved by Research Ethics Committee of the Hospital de la Santa Creu I Sant Pau, Barcelona, Spain (Comitè d'Ètica d'Investigació amb Medicaments, CEIm). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

MS: Writing – original draft, Writing – review & editing, Validation, Data curation, Formal analysis, Investigation, Methodology. JI: Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. FP: Investigation, Validation, Writing – original draft, Writing – review & editing, Supervision. JG: Supervision, Validation, Writing – original draft, Writing – review & editing, Conceptualization. AA: Supervision, Writing – original draft, Writing – review & editing. LM: Writing – original draft, Writing – review & editing, Investigation, Methodology. SW: Writing – original draft, Writing – review & editing, Supervision, Validation. EV: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

JSI-C was employed by company EpiDisease S.L. JLG-G is a founding partner of EpiDisease SL, a Spin-off from the Center for Biomedical Network Research of Spain.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1410080/full#supplementary-material

SUPPLEMENTARY TABLE 1
Identification of differentially expressed miRNAs by small RNA-sequencing.

SUPPLEMENTARY TABLE 2
Over-representation analysis using Gene Ontology terms to identify pathways significantly regulated by the identified miRNAs (miR-28-5p, miR-495-3p and miR-654-5p).

SUPPLEMENTARY TABLE 3
Over-representation analysis using the Kyoto Encyclopaedia of Genes and Genomes to identify pathways significantly regulated by the identified miRNAs (miR-28-5p, miR-495-3p and miR-654-5p).

References

1. Valassi E, Santos A, Yaneva M, Tóth M, Strasburger CJ, Chanson P, et al. The European Registry on Cushing's syndrome: 2-year experience. Baseline demographic and clinical characteristics. *Eur J Endocrinol*. (2011) 165:383–92. doi: 10.1530/EJE-11-0272

2. Valassi E, Crespo I, Santos A, Webb SM. Clinical consequences of Cushing's syndrome. *Pituitary*. (2012) 15:319–29. doi: 10.1007/s11102-012-0394-8

3. Vogel J, Braun LT, Rubinstein G, Zopp S, Künzel H, Strasding F, et al. Persisting muscle dysfunction in Cushing's syndrome despite biochemical remission. *J Clin Endocrinol Metab*. (2020) 105:e4490–8. doi: 10.1210/clinem/dgaa625

4. Schakman O, Gilson H, Thissen JP. Mechanisms of glucocorticoid induced myopathy. *J Endocrinol*. (2008) 197:1–10. doi: 10.1677/JOE-07-0606

5. Wajchenberg BL, Bosco A, Marone MM, Levin S, Rocha M, Lerário AC, et al. Estimation of body fat and lean tissue distribution by dual energy X-ray absorptiometry and abdominal body fat evaluation by computed tomography in Cushing's disease. *J Clin Endocrinol Metab*. (1995) 80:2791–94. doi: 10.1210/jcem.80.9.7673425

6. Geer EB, Shen W, Gallagher D, Punyanitya M, Looker HC, Post KD, et al. MRI assessment of lean and adipose tissue distribution in female patients with Cushing's disease. *Clin Endocrinol (Oxf)*. (2010) 73:469–75. doi: 10.1111/j.1365-2265.2010.03829.x

7. Martel-Duguech L, Alonso-Jiménez A, Bascuñana H, Díaz-Manera J, Llauger J, Nuñez-Peralta C, et al. Thigh muscle fat infiltration is associated with impaired physical performance despite remission in Cushing's syndrome. *J Clin Endocrinol Metab*. (2020) 105:2039–49. doi: 10.1210/clinem/dgz329

8. Berr CM, Stieg MR, Deutschbein T, Quinkler M, Schmidmaier R, Osswald A, et al. Persistence of myopathy in Cushing's syndrome: evaluation of the German Cushing's Registry. *Eur J Endocrinol*. (2017) 176:737–46. doi: 10.1530/EJE-16-0689

9. Studenski SA, Peters KW, Alley DE, Cawthon PM, McLean RR, Harris TB, et al. The FNIH sarcopenia project: rationale, study description, conference recommendations, and final estimates. *J Gerontol A Biol Sci Med Sci*. (2014) 69:547–58. doi: 10.1093/gerona/glu010

10. Martel-Duguech L, Alonso-Jiménez A, Bascuñana H, Díaz-Manera J, Llauger J, Nuñez-Peralta C, et al. Prevalence of sarcopenia after remission of hypercortisolism and its impact on HRQoL. *Clin Endocrinol*. (2021) 95:735–43. doi: 10.1111/cen.14568

11. Widmann M, Niess AM, Munz B. Physical exercise and epigenetic modifications in skeletal muscle. *Sports Med*. (2019) 49:509–23. doi: 10.1007/s40279-019-01070-4

12. Peiró-Chova L, Ponce OB, Abril-Tormo C, Martínez-Santamaría J, López-Guerrero JA, Riegman PHJ. Chapter 2: the importance of biobanks in epigenetic studies. In: García-Giménez JL, editor. *Epigenetic Biomarkers and Diagnostics*. Academic Press, Boston (2016). p. 19–35.

13. Brusa R, Magri F, Bresolin N, Comi GP, Corti S. Noncoding RNAs in Duchenne and Becker muscular dystrophies: role in pathogenesis and future prognostic and therapeutic perspectives. *Cell Mol Life Sci*. (2020) 77:4299–313. doi: 10.1007/s00018-020-03537-4

14. Siracusa J, Koulmann N, Banzet S. Circulating myomiRs: a new class of biomarkers to monitor skeletal muscle in physiology and medicine. *J Cachexia Sarcopenia Muscle*. (2018) 9:20–7. doi: 10.1002/jcsm.12227

15. Rusanova I, Fernández-Martínez J, Fernández-Ortiz M, Aranda-Martínez P, Escames G, García-García FJ, et al. Involvement of plasma miRNAs, muscle miRNAs and mitochondrial miRNAs in the pathophysiology of frailty. *Exp Gerontol*. (2019) 124:110637. doi: 10.1016/j.exger.2019.110637

16. Carrasco-Rozas A, Fernández-Simón E, Lleixa MC, Belmonte I, Pedrosa-Hernández I, Montiel-Morillo E, et al. Identification of serum microRNAs as potential biomarkers in Pompe disease. *Ann Clin Translat Neurol*. (2019) 6:1214–24. doi: 10.1002/acn3.50800

17. Lutz CT, Quinn LS. Sarcopenia, obesity, and natural killer cell immune senescence in aging: altered cytokine levels as a common mechanism. *Aging (Albany NY)*. (2012) 4:535–46. doi: 10.18632/aging.v4i8

18. Chen FX, Du N, Hu J, Ning F, Mei X, Li Q, et al. Intramuscular accumulation of pentadecanoic acid activates AKT1 to phosphorylate NCOR1 and triggers FOXM1-mediated apoptosis in the pathogenesis of sarcopenia. *Am J Transl Res*. (2020) 12(9):5064–79.

19. Englund DA, Zhang X, Aversa Z, Le Brasseur NK. Skeletal muscle aging, cellular senescence, and senotherapeutics: Current knowledge and future directions. *Mech Ageing Dev*. (2021) 200:111595. doi: 10.1016/j.mad.2021.111595

20. He Y, Xie W, Li H, Jin H, Zhang Y, Li Y. Cellular senescence in sarcopenia: possible mechanisms and therapeutic potential. *Front Cell Dev Biol*. (2021) 9:793088. doi: 10.3389/fcell.2021.793088

21. Hata R, Miyamoto K, Abe Y, Sasaki T, Oguma Y, Tajima T, et al. Osteoporosis and sarcopenia are associated with each other and reduced IGF1 levels are a risk for both diseases in the very old elderly. *Bone*. (2023) 166:116570. doi: 10.1016/j.bone.2022.116570

22. Ascenzi F, Barberi L, Dobrowolny G, Villa Nova Bacurau A, Nicoletti C, Rizzuto E, et al. Effects of IGF-1 isoforms on muscle growth and sarcopenia. *Aging Cell*. (2019) 18:e12954. doi: 10.1111/acel.12954

23. Pivonello C, Patalano R, Simeoli C, Monto T, Negri M, Amatrudo F, et al. Circulating myomiRNAs as biomarkers in patients with Cushing's syndrome. *J Endocrinol Invest*. (2024) 47:655–69. doi: 10.1007/s40618-023-02184-3

24. Nieman LK, Biller BM, Findling JW, Newell-Price J, Savage MO, Stewart PM, et al. The diagnosis of Cushing's syndrome: an Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab*. (2008) 93:1526–40. doi: 10.1210/jc.2008-0125

25. Vlachos IS, Zagganas K, Paraskevopoulou MD, Georgakilas G, Karagkouni D, Vergoulis T, et al. DIANA-miRPath v3.0: deciphering microRNA function with experimental support. *Nucleic Acids Res*. (2015) 43:W460–466. doi: 10.1093/nar/gkv403

26. Szpechcinski A, Florczuk, Duk MK, Zdral A, Rudzinski S, Bryl M, et al. The expression of circulating miR-504 in plasma is associated with EGFR mutation status in non-small-cell lung carcinoma patients. *Cell Mol Life Sci*. (2019) 76:3641–56. doi: 10.1007/s00018-019-03089-2

27. García-Giménez JL, Rubio-Belmar PA, Peiró-Chova L, Hervás D, González-Rodríguez D, Ibánez-Cabellos JS, et al. Circulating miRNAs as diagnostic biomarkers for adolescent idiopathic scoliosis. *Sci Rep*. (2018) 8:2646. doi: 10.1038/s41598-018-21146-x

28. Cardo L, Coto FE, de Mena L, Ribacoba R, Moris G, Menéndez M, et al. Profile of microRNAs in the plasma of Parkinson's disease patients and healthy controls. *J Neurol*. (2013) 260:1420–22. doi: 10.1007/s00415-013-6900-8

29. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. (2001) 25:402–8. doi: 10.1006/meth.2001.1262

30. Håkansson KEJ, Sollie O, Simons KH, Quax PHA, Jensen J, Nossent AY. Circulating small non-coding RNAs as biomarkers for recovery after exhaustive or repetitive exercise. *Front Physiol*. (2018) 9:1136. doi: 10.3389/fphys.2018.01136

31. Mir BA, Islam R, Kalanon M, Russell AP, Foletta VC. MicroRNA suppression of stress-responsive NDRG2 during dexamethasone treatment in skeletal muscle cells. *BMC Mol Cell Biol*. (2019) 20:12. doi: 10.1186/s12860-019-0194-3

32. Williamson D, Gallagher P, Harber M, Hollon C, Trappe S. Mitogen-activated protein kinase (MAPK) pathway activation: Effects of age and acute exercise on human skeletal muscle. *J Physiol*. (2003) 547:977–87. doi: 10.1113/jphysiol.2002.036673

33. Mylabathula DB, Rice KM, Wang Z, Uddemarri S, Kinnard RS, Blough ER. Age-associated changes in MAPK activation in fast- and slow-twitch skeletal muscle of the F344/NNiaHSD X Brown Norway/BiNia rat model. *Exp Gerontol*. (2006) 41:205–14. doi: 10.1016/j.exger.2005.11.001

34. Filomena MC, Yamamoto DL, Caremani M, Kadarla VK, Mastrototaro G, Serio S, et al. Myopalladin promotes muscle growth through modulation of the serum response factor pathway. *J Cachexia Sarcopenia Muscle*. (2020) 11:169–94. doi: 10.1002/jcsm.12486

35. Shang GK, Han L, Wang ZH, Liu YP, Yan SB, Sai WW, et al. Sarcopenia is attenuated by TRB3 knockout in aging mice via the alleviation of atrophy and fibrosis of skeletal muscles. *J Cachexia Sarcopenia Muscle*. (2020) 11:1104–20. doi: 10.1002/jcsm.12560

36. Djemai H, Hassani M, Daou N, Li Z, Sotiropoulos A, Noirez P, et al. Srf KO and wild-type mice similarly adapt to endurance exercise. *Eur J Transl Myol*. (2019) 29:8205. doi: 10.4081/ejtm.2019.8205

37. Nakamura S, Sato Y, Kobayashi T, Oike T, Kaneko Y, Miyamoto K, et al. Insulin-like growth factor-I is required to maintain muscle volume in adult mice. *J Bone Miner Metab*. (2019) 37:627–35. doi: 10.1007/s00774-018-0964-6

38. Tortorella LL, Milasincic DJ, Pilch PF. Critical proliferation-independent window for basic fibroblast growth factor repression of myogenesis via the p42/p44

MAPK signaling pathway. *J Biol Chem*. (2001) 276:13709–17. doi: 10.1074/jbc.M100091200

39. Fan Y, Zhang Z, Deng K, Kang Z, Guo J, Zhang G, et al. CircUBE3A promotes myoblasts proliferation and differentiation by sponging miR-28-5p to enhance expression. *Int J Biol Macromol*. (2023) 226:730–45. doi: 10.1016/j.ijbiomac.2022.12.064

40. Dmitriev P, Barat A, Polesskaya A, O'Connell MJ, Robert T, Dessen TA, et al. Simultaneous miRNA and mRNA transcriptome profiling of human myoblasts reveals a novel set of myogenic differentiation-associated miRNAs and their target genes. *BMC Genomics*. (2013) 14:265. doi: 10.1186/1471-2164-14-265

41. Macsue J, Hiam D, Craig J, Barrès R, Eynon N, Voisin S. Epigenetic changes in healthy human skeletal muscle following exercise– a systematic review. *Epigenetics*. (2019) 14:633–48. doi: 10.1080/15592294.2019.1614416

42. Keller P, Vollaard NBJ, Gustafsson T, Gallagher IJ, Sundberg CJ, Rankinen T, et al. A transcriptional map of the impact of endurance exercise training on skeletal muscle phenotype. *J Appl Physiol*. (2011) 110:46–59. doi: 10.1152/japplphysiol.00634.2010

43. Huang DD, Fan SD, Chen XY, Yan XL, Zhang XZ, Ma BW, et al. Nrf2 deficiency exacerbates frailty and sarcopenia by impairing skeletal muscle mitochondrial biogenesis and dynamics in an age-dependent manner. *Exp Gerontol*. (2019) 119:61–73. doi: 10.1016/j.exger.2019.01.022

44. Yokoyama S, Ito Y, Ueno-Kudoh H, Shimizu H, Uchibe K, Albini S, et al. A systems approach reveals that the myogenesis genome network is regulated by the transcriptional repressor RP58. *Dev Cell*. (2009) 17:836–48. doi: 10.1016/j.devcel.2009.10.011

45. Cushing H. The basophil adenomas of the pituitary body and their clinical manifestations (pituitary basophilism). *Obes Res*. (1932) 2:486–508. doi: 10.1002/j.1550-8528.1994.tb00097.x

46. Liu Q, Yan S, Yuan Y, Ji S, Guo L. miR-28-5p improved carotid artery stenosis by regulating vascular smooth muscle cell proliferation and migration. *Vascular*. (2022) 30:764–70. doi: 10.1177/17085381211019510

47. Lee RS, Zandi PP, Santos A, Aulinas A, Carey JL, Webb SM, et al. Cross-species association between telomere length and glucocorticoid exposure. *J Clin Endocrinol Metab*. (2021) 106:e5124–e35. doi: 10.1210/clinem/dgab519

48. Aulinas A, Ramírez MJ, Barahona MJ, Valassi E, Resmini E, Mato E, et al. Dyslipidemia and chronic inflammation markers are correlated with telomere length shortening in Cushing's syndrome. *PloS One*. (2015) 10:e0120185. doi: 10.1371/journal.pone.0120185

49. Barahona MJ, Sucunza N, Resmini E, Fernández-Real JM, Ricart W, Moreno-Navarrete JM, et al. Persistent body fat mass and inflammatory marker increases after long-term cure of Cushing's syndrome. *J Clin Endocrinol Metab*. (2009) 94:3365–71. doi: 10.1210/jc.2009-0766

50. De Larichaudy J, Zufferli A, Serra F, Isidori AM, Naro F, Dessalle K, et al. TNF-alpha- and tumor-induced skeletal muscle atrophy involves sphingolipid metabolism. *Skelet Muscle*. (2012) 2:2. doi: 10.1186/2044-5040-2-2

51. Vetrivel S, Zhang R, Engel M, Altieri B, Braun L, Osswald A, et al. Circulating microRNA expression in Cushing's syndrome. *Front Endocrinol*. (2021) 12:620012. doi: 10.3389/fendo.2021.620012

52. Dogra P, Šambula L, Saini J, Thangamuthu K, Athimulam S, Delivanis DA, et al. High prevalence of frailty in patients with adrenal adenomas and adrenocortical hormone excess: a cross-sectional multi-centre study with prospective enrolment. *Eur J Endocrinol*. (2023) 189:318–26. doi: 10.1093/ejendo/lvad113

53. Liu L, Wang S, Wang M, Liu G, Yang Z, Wang L. 1miR-654-5p suppresses migration and proliferation of vascular smooth muscle cells by targeting ADAMTS-7. *Cells Tissues Organs*. (2023) 212:285–92. doi: 10.1159/000524677

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Impacts of dietary animal and plant protein on weight and glycemic control in health, obesity and type 2 diabetes: friend or foe?

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It is well established that high-protein diets (i.e. ~25–30% of energy intake from protein) provide benefits for achieving weight loss, and subsequent weight maintenance, in individuals with obesity, and improve glycemic control in type 2 diabetes (T2D). These effects may be attributable to the superior satiating property of protein, at least in part, through stimulation of both gastrointestinal (GI) mechanisms by protein, involving GI hormone release and slowing of gastric emptying, as well as post-absorptive mechanisms facilitated by circulating amino acids. In contrast, there is evidence that the beneficial effects of greater protein intake on body weight and glycemia may only be sustained for 6–12 months. While both suboptimal dietary compliance and metabolic adaptation, as well as substantial limitations in the design of longer-term studies are all likely to contribute to this contradiction, the source of dietary protein (i.e. animal vs. plant) has received inappropriately little attention. This issue has been highlighted by outcomes of recent epidemiological studies indicating that long-term consumption of animal-based protein may have adverse effects in relation to the development of obesity and T2D, while plant-based protein showed either protective or neutral effects. This review examines information relating to the effects of dietary protein on appetite, energy intake and postprandial glycemia, and the relevant GI functions, as reported in acute, intermediate- and long-term studies in humans. We also evaluate knowledge relating to the relevance of the dietary protein source, specifically animal or plant, to the prevention, and management, of obesity and T2D.

KEYWORDS

animal protein, appetite, food intake, gastrointestinal function, glycemic control, obesity, plant protein, type 2 diabetes

1 Introduction

In the last ~20 years, there has been substantial, and increasing, interest in promoting dietary protein intake to improve health outcomes (1–5). We believe that the first official recommendation for daily protein intake, reported in 1936 by the League of Nations (6), was 1.0 g/kg of body weight. This has been subsequently challenged by several joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) expert committees, who made the current recommendation of 0.8 g/kg daily protein intake in healthy adults, accounting for ~10–15% of daily energy intake, in 2007 (7). There is now compelling evidence that high-protein diets, which can entail a protein intake up to 5-fold greater than the recommended daily amount, and are in most cases characterized by ~25–30% of energy intake from protein, facilitate weight loss and attenuate weight (re)gain, in individuals with obesity, and improve glycemic control in type 2 diabetes (T2D), in the intermediate-term, i.e. during 6–12 months' consumption (8–10). Protein suppresses energy intake (11–16), and reduces postprandial glycemia (17–20). These effects may be attributable to the capacity of protein to stimulate both gastrointestinal (GI) hormones (11–13) and postabsorptive, possibly 'central', mechanisms in response to meals (21, 22). Key GI hormones include cholecystokinin (CCK), the so-called 'incretin' hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1), as well as peptide tyrosine-tyrosine (PYY), which are pivotal to the regulation of both energy intake and/or postprandial blood glucose, in some cases, at least in part, through slowing of gastric emptying (23–25) (Figure 1).

In contrast to these potent acute/intermediate-term effects of protein, there is evidence that the beneficial effects of greater protein intake on body weight and glycemia may only be sustained for 6–12 months (26–28), which has been attributed to both suboptimal dietary compliance and metabolic adaptation. However, the substantial variations, as well as limitations in the design of longer-term studies, including inconsistencies in the amount and composition of tested foods, and the characteristics of study participants (e.g. age, ethnicity and race) are also likely to be relevant. There are also considerable variations in the source of dietary protein between individuals worldwide (29, 30), which may be derived from animal- and/or plant-based foods. This issue has received less attention despite compelling evidence that animal and plant proteins may have different metabolic effects in the longer-term (31–33). This issue has assumed increasing importance, particularly in view of emerging evidence derived from recent epidemiological studies to indicate an increased risk of T2D with animal, but a protective effect of plant, protein (34–36).

The focus of this review relates to the effects of dietary protein on appetite, energy intake and postprandial glycemia, and the relevant GI functions, including the stimulation of GI hormones and slowing of gastric emptying, as reported in acute, intermediate- and long-term studies in humans. We also evaluate knowledge

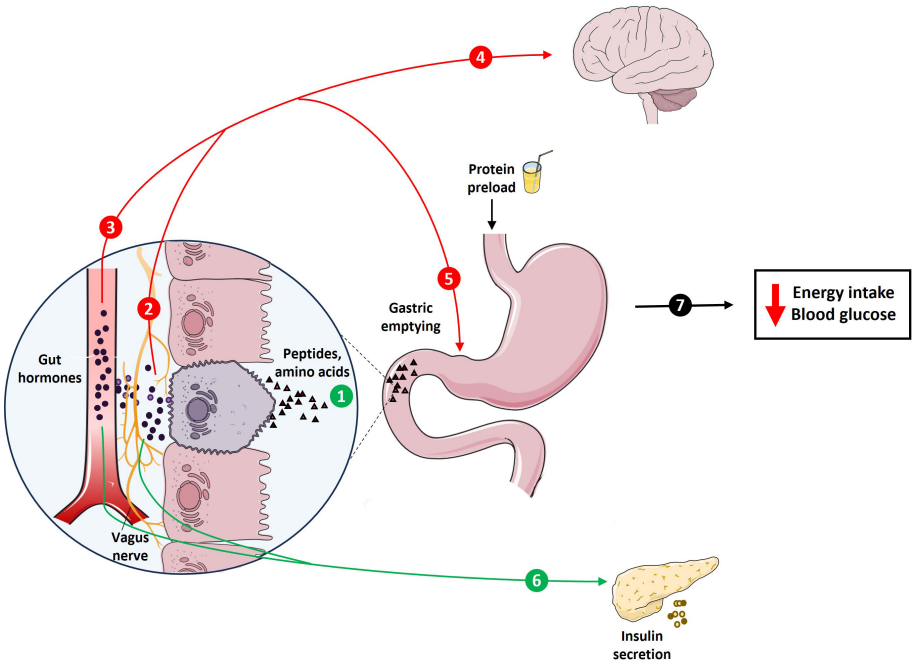

FIGURE 1
Schematic representation of protein-induced stimulation of gastrointestinal (GI) functions, including GI hormone release and slowing of gastric emptying, which are integral to the regulation of energy intake and glycemia. The presence of protein digestion products, including peptides and amino acids, in the GI lumen stimulates key GI hormones, including CCK, the incretins, GIP and GLP-1, and PYY (1). These hormones exert their effects through various pathways, including activation of hormone-specific receptors on vagal afferent endings (2) or following transport through the bloodstream (3). These inputs, together, are conveyed to higher brain centers to modulate eating behaviors (4), as well as feedback regulation of GI motor functions, particularly stimulation of pyloric pressures, associated with the slowing of gastric emptying (5). GIP and GLP-1, when transported in the bloodstream and/or by activating receptors on vagal afferent endings, also stimulate insulin secretion from pancreas (6). Together, these signals contribute to the effects of protein to reduce energy intake and blood glucose (7).

relating to the relevance of the dietary protein source, specifically animal or plant, to the prevention, and management, of obesity and T2D. While dietary protein is also of importance to other areas, including muscle mass, particularly in elderly and malnourished people, these and other issues are not addressed.

2 Acute effects of protein on appetite, energy intake and postprandial glycemia

A number of studies have shown that acute oral administration of protein preloads, in doses ranging from 20–70 g, dose-dependently reduce hunger, and induce fullness, associated with suppression of energy intake at a subsequent meal, in both healthy lean individuals and those with obesity (11–16, 20, 37). A comprehensive meta-analysis comprising 49 trials, which investigated the acute effects of protein preloads on commonly used markers of appetite, revealed decreases in hunger, desire to eat, prospective food consumption, and an increase in fullness in both lean and obese participants (38). These effects were associated with a reduction in subsequent food intake, when participants were presented with a standardized meal (38). These effects of protein are also often accompanied by reductions in postprandial glycemia. Indeed, a higher protein intake, either as a 'preload' before, or as part of, a carbohydrate meal, has been shown to reduce postprandial glycemic excursions, in both lean and obese individuals with and without T2D (10, 13, 17–20, 39–42). Accordingly, the outcomes of these acute studies are consistent, showing that a higher protein intake at a meal has beneficial effects to reduce both energy intake and postprandial glycemia.

The acute appetite- and glucoregulatory effects of protein have been shown to vary between different sources of protein (43–59) (Table 1). For example, when the effects of preloads, containing either milk proteins (whey or casein protein), egg, turkey, tuna, or soy protein, were compared, each suppressed hunger and energy intake, but whey protein had the most profound effects (44, 49, 51, 53, 56). In contrast, a number of studies reported that whey protein was less satiating than some other proteins (46, 47, 55). For example, when the effects of whey protein, pea protein hydrolysate, a combination of whey protein and pea protein hydrolysate, and control milk protein (80% casein and 20% whey) were compared, pea protein hydrolysate was the most effective in suppressing hunger and desire to eat, with no difference in their effects on subsequent energy intake (46). Veldhorst et al. also reported that both alpha-lactalbumin and gelatin are ~40% more satiating than whey protein, inducing a related ~20% reduction in subsequent energy intake (55). However, when compared with casein or soy, whey protein was still more effective in suppressing energy intake (56). Milk proteins have also been found, in some studies, to exert more potent effects to reduce blood glucose than turkey, fish, egg, or pea proteins (52–54, 57). For example, when 45 g of protein, of different sources (either gluten, cod, casein, or whey), was added to a high-fat meal, the postprandial blood glucose response in T2D was less with whey, compared to the other proteins (52). In contrast, in another study in healthy and prediabetic adults, there was no difference in postprandial glycemic excursions between whey and casein, when added to a drink containing maltodextrin (50). Whether variations in the effects of different

TABLE 1 Acute effects of different protein preloads (animal vs. plant) on ad-libitum energy intake and postprandial blood glucose levels.

First author	Country	Design	Sample size (n)	Age (y)	BMI (kg/m^2)	Health status	Protein source	Protein dose	Duration[1] (min)	EI[2] (kcal)	BG[3]	Ref
Hall et al. (2003)	UKD	Cross-over	16 (M/F)	22	21.7	Healthy lean	Casein Whey	48 g	90	1084 878	NR	(49)
Anderson et al. (2004)	Canada	Cross-over	13 (M)	22	22	Healthy lean	Egg Soy Whey	0.65 g/kg (~46 g)	60	912 729 661	NR	(44)
Nilsson et al. (2004)	Sweden	Cross-over	12 (M/F)	20-28	21.9	Healthy lean	Gluten Cod Cheese Milk Whey	18.2 g	90	NR	35.4* 43.9 39.3 19.3 21.8	(57)
Bowen et al. (2006)	Australia	Cross-over	72 (M)	50-56	23-30	Healthy lean and overweight	Soy Gluten Whey	50 g 51 g 51 g	180	766 718 769	5.8 5.9 5.9	(45)
Diepvens et al. (2008)	The Netherlands	Cross-over	39 (M/F)	42	27.6	Overweight	Pea Whey Pea + Whey	15 g	180	304 299 309	NR	(46)
Veldhorst et al. (2009)	The Netherlands	Cross-over	25 (M/F)	22	23.9	Healthy lean	Soy Casein Whey Gelatin	25% of high-protein custard (~40 g)	180	767 736 687	122* 68 95	(55, 56)

(Continued)

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TABLE 1 Continued

First author	Country	Design	Sample size (n)	Age (y)	BMI (kg/m^2)	Health status	Protein source	Protein dose	Duration[1] (min)	EI[2] (kcal)	BG[3]	Ref
							Alpha-lactalbumin			556 501	82 84	
Mortensen et al. (2009)	Denmark	Cross-over	12 (M/F)	64	28.9	T2D	Gluten Cod Casein Whey	45 g	480	NR	495* 396 375 233	(52)
Pal et al. (2010)	Australia	Cross-over	22 (M)	23	22.6	Healthy lean	Egg Turkey Tuna Whey	50.8 g	240	844 839 782 705	5.45 5.49 5.39 4.59	(53)
Acheson et al. (2011)	Switzerland	Cross-over	23 (M/F)	32	22.7	Healthy lean	Soy Casein Whey	0.81 g/kg (~56.7 g)	330	NR	5.9 6.1 6.1	(43)
Gunnerud et al. (2012)	Sweden	Cross-over	14 (M/F)	20-28	21.9	Healthy lean	Soy Whey	9 g	60	NR	60.6* 54.7	(48)
Teunissen-Beekman et al. (2014)	The Netherlands	Cross-over	48 (M/F)	58	28.6	Overweight or obesity	Egg Pea Milk	0.6 g/kg (~70 g)	240	NR	-3.8** -3.8 -4.2	(54)
Hoefle et al. (2015)	Germany	Cross-over	15 (M) 15 (M/F)	26 62	23.9 29	Healthy lean Prediabetes	Casein Whey Casein Whey	50 g	240	NR	5.7 5.4 7.5 7.7	(50)
Dougkas et al. (2018)	Sweden	Cross-over	28 (M)	28	23.4	Healthy lean	Plant proteins (oat, pea and potato) Milk proteins 50:50 mixture	25% of high-protein pudding (~25 g)	210	760 816 795	7.5 7.9 7.5	(47)
Melson et al. (2019)	USA	Cross-over	17 (M/F)	27	24.6	Healthy lean	Soy Whey	50 g 43.3 g	180	664 654	NR	(51)

M, Male; F, Female; Y, Years; BMI, Body mass index; EI, Energy intake; BG, Blood glucose; NR, Not reported; T2D, Type 2 diabetes.
[1] Time interval between protein preload and an ad-libitum test meal; [2] Energy intake at an ad-libitum test meal; [3] Reported as peak concentration of postprandial glucose in mmol/L, otherwise indicated as areas under the curve (AUCs) (mmol/L.h*) or changes from baseline (mmol/L**).

protein sources to reduce postprandial glycemia are associated with the magnitude of their effect on appetite remains uncertain, with some studies suggesting a strong relationship, particularly for whey (43, 53, 60). The latter is potentially attributable to the rapid digestion of whey protein, due to its high solubility in the acidic environment of the stomach, leading to the stimulation of GI mechanisms more effectively than other proteins (49, 60). However, comprehensive evidence comparing all types of protein sources, particularly different plant-based proteins, is lacking and further investigation is required.

2.1 Mechanisms underlying the effects of protein on energy intake and glycemia

The stimulation of both GI mechanisms, involving GI hormone release and slowing of gastric emptying (23–25), as well as post-absorptive mechanisms facilitated by specific circulating amino acids (21, 22), have been shown to be integral to these effects of protein. Protein, and its digestion products (amino acids), when administered directly into the GI lumen, stimulate key GI hormones, including CCK, the incretins, GIP and GLP-1, and PYY (61–70). In addition to the direct activation of receptors on submucosal vagal afferent and enteric neurons to modulate eating behavior (71), these hormones are transported in the bloodstream to affect peripheral organs, including the stomach, to stimulate pyloric pressures, which are important to the regulation of gastric emptying, and the pancreas, to stimulate insulin secretion (72), overall resulting in a reduction in postprandial glycemia (Figure 1). The rate of gastric emptying plays a key role in determining the postprandial glycemic response, particularly in the first 30–60 min following a meal, accounting for up to 35% of the variance in the initial glycemic response to a meal in healthy participants (73). With progressive impairment in glucose tolerance, this relationship exhibits a ‘shift to the right’, so that the 120-min blood glucose in a

75 g oral glucose tolerance test is inversely related to the rate of gastric emptying in healthy participants, but directly in people with T2D (74). Proteins also stimulate glucagon secretion, which may affect postprandial glycemia adversely (75). Moreover, postprandial glucagon secretion is characteristically exaggerated in individuals with T2D (76).

As alluded to, these acute effects of protein to modulate GI functions are dependent on the type of protein, with evidence that whey protein is more potent than other protein sources, including casein, fish, soy, gluten and pea protein (45, 46, 48, 49, 53, 56, 57). Accordingly, the majority of studies have focused predominantly on whey protein. For example, in healthy men, a 60-min intraduodenal infusion of whey protein, in loads of 0.5, 1.5 and 3 kcal/min, reflecting the physiological range of gastric emptying of ~1–3 kcal/min, has been shown to stimulate plasma CCK and GLP-1 concentrations, and pyloric pressures, all in a dose-dependent manner, associated with suppression of subsequent energy intake in both lean men (61) and those with obesity (62). At the highest load (3 kcal/min), whey protein also reduced blood glucose levels in individuals without T2D (61, 62). Oral preloads of whey protein, in doses of 30 and 70 g, also stimulated plasma CCK, GLP-1, glucagon, and slowed gastric emptying, associated with suppression of energy intake, and improved glycemia, in healthy men (13). In T2D, acute administration of whey protein, ingested as a preload, in a dose of 55 g, 30 min before a mashed potato meal, also stimulated GLP-1, GIP and insulin and slowed gastric emptying, associated with a substantial reduction in peak postprandial glucose of ~3 mmol/L (17). Moreover, these effects were shown to be sustained when whey protein (25 g) was given 30 min before each of three main meals, for 4 weeks (18). Similar acute effects of whey protein were evident when a preload incorporating whey (17 g) together with guar (5 g), a viscous polysaccharide that can itself reduce postprandial glycemic excursions, was given to individuals with T2D or prediabetes (77). 12 weeks' treatment with this preload, consumed twice daily before breakfast and dinner in individuals with well-controlled T2D, had sustained effects to slow gastric emptying and reduce postprandial blood glucose (78).

There is evidence that the high content of branched-chain amino-acids (BCAAs), particularly leucine and isoleucine, in whey protein, contributes to its efficacy in reducing energy intake and glycemia, through stimulation of GI hormone secretion (57, 79, 80). For example, intraduodenal administration of L-leucine, in a load of 0.45 kcal/min (9.9 g over 90 min), stimulated CCK secretion and suppressed subsequent energy intake (63). Moreover, both L-leucine and L-isoleucine, when administered intragastrically, in a dose of 10 g, 30 min before a mixed-nutrient drink (500 kcal), lowered postprandial blood glucose (67). In contrast, valine was ineffective, potentially reflecting the concurrent stimulation of glucagon (67). L-leucine also stimulated C-peptide, a marker of insulin secretion, and both L-leucine and L-isoleucine slowed gastric emptying of the drink modestly (67). However, these effects of L-leucine and L-isoleucine were not evident in individuals with T2D for uncertain reasons (68). There is also compelling evidence that these effects of amino acids are type-specific, with some, such as L-proline (81), and L-lysine (82), being less potent, compared to the aromatic amino acids, L-tryptophan (65, 66, 83) and L-phenylalanine (69). In a comparative analysis of the effects of four different amino acids (L-tryptophan, L-phenylalanine, L-leucine and L-glutamine) administered intraduodenally, L-tryptophan and L-leucine were shown to have the most potent effects to reduce energy intake, which was related to greater stimulation of plasma CCK (70). Both L-tryptophan (dose of 3 g) (66) and L-phenylalanine (dose of 10 g) (69), when administered intragastrically before a carbohydrate-containing drink, also lowered the blood glucose response in healthy lean participants (66, 69) and those with obesity (66).

The concept that these amino acids may also mediate the effects of dietary protein, after absorption, via both vagal mechanisms and direct effects on specific brain regions, including the hypothalamus and brainstem (21, 22, 84), was first introduced in 1956, as the so-called 'aminostatic hypothesis', which recognized that while amino acids are used primarily for protein synthesis, the amino acids remaining in the circulation might serve as a food intake-regulatory signal (84). BCAAs, particularly L-leucine, were shown to activate the mammalian target of rapamycin complex 1 (mTORC1), to act as a cellular fuel sensor in which hypothalamic activity is tied directly to the regulation of energy intake. In a variety of model systems, mTOR activity has been shown to be highly sensitive to plasma levels of L-leucine (85, 86). There is also emerging preclinical evidence to support a major role for BCAAs, particularly L-leucine and L-isoleucine, in β-cell signaling and metabolism, to acutely stimulate insulin secretion through activation of mTORC1, which is also responsible for increasing β-cell mass and function (87, 88). Elevated plasma concentrations of other amino acids, particularly L-tryptophan, which serves as a precursor for the neurotransmitter serotonin, a key regulator of appetite (89), have also been reported to be associated with reduced energy intake. A lesser number of studies have addressed the role of other amino acids. Both tyrosine and histidine can be converted into anorexigenic neurotransmitters, including dopamine, norepinephrine and histamine, but their contributions to protein-induced food intake suppression remain uncertain (90, 91). Thus, amino acids appear to mediate, at least in part, the effects of protein through distinct physiological pathways. This is likely to be important given that the amino acid composition of different sources of proteins may represent a major factor to account for their diverse metabolic effects in the longer-term.

3 Intermediate-term effects of protein on food intake, body weight and glycemia

The capacity of high-protein diets to induce weight loss has been examined primarily through two approaches; 'ad-libitum' diets, in which participants are allowed to consume based on their desire to eat, and 'energy-restricted' diets, where the proportion of protein is increased while restricting and then maintaining a constant total energy intake. Irrespective of the type of dietary protocol, in a majority of studies, enriching diets with a relatively high protein content has been shown to facilitate

weight loss more than standard-protein diets (~10–15% of energy intake from protein) with intervention durations of up to 6 months (92–96). Ad-libitum high-protein diets, however, have shown more consistent efficacy, while under iso-energetic conditions, strict control of energy intake has invariably been associated with clinically relevant weight loss that compromised assessment of potential metabolic effects of protein. A number of meta-analyses have reported favorable effects of high-protein diets on weight loss (8, 9, 97). For example, a meta-analysis of 24 randomized clinical trials that only compared energy-restricted isocaloric high-protein (27–35% protein) and standard-protein (16–21% protein) diets, with a mean diet duration of 12 weeks, revealed modestly greater reductions in weight (-0.79 kg) and fat mass (-0.87 kg) with a high-protein diet (8). Another meta-analysis of 74 randomized controlled trials, investigating the effects of high-protein diets with or without energy restriction, with a mean duration of 6 months, also found reductions in body weight (-0.36 kg), body mass index (-0.37 kg/m^2), and waist circumference (-0.43 cm) in the high-protein (16–45% protein) compared to the standard-protein (5–23% protein) diet group (97).

In contrast to the promising and relatively consistent outcomes of the shorter-term effects (≤6 months duration) of high-protein diets on weight loss in numerous studies, the majority of longer-term studies (at least 12 months in duration), albeit much fewer in number, found no effect of higher protein intake (26–28, 98–102). For example, in a follow-up to an intensive 6-month weight-loss trial, Due et al. reported that, at 12 months, weight loss was no greater in participants assigned to a high-protein diet (30% protein), compared with a medium-protein diet (12% protein) (98). A 2013 meta-analysis, which included 15 trials, in which the intervention period was for a minimum of 12 months, also revealed neither a beneficial, nor detrimental, effect of higher protein intakes on weight loss (28). In contrast, in a 12-month study, McAuley et al. reported modestly improved weight-loss maintenance (-6.6 kg) with a higher-protein diet (30% protein) than either a high-carbohydrate diet (-4.4 kg) or a high-fat diet (-5.5 kg), each containing 15% protein (99). Clifton et al. also found a direct relationship between weight loss and protein intake when comparing high-protein (34% protein) with high-carbohydrate diets (containing 17% protein) for 12 months, however, there was no difference in weight loss effects of the two diets (101). In two trials by Brinkworth et al, one in people with T2D (27), the other in normoglycemic individuals with obesity (26), the effects of a high-protein diet (30% protein) and a standard-protein diet (15% protein), both low in fat, during 8 to 12 weeks of energy restriction and 12 months of energy balance were compared, reporting a net weight loss in both groups, which was slightly greater in the high-protein group (-3.7 to -4.1 kg) compared with the standard-protein group (-2.2 to -2.9 kg). Accordingly, while the majority of evidence indicates that the efficacy of high-protein diets is attenuated in the longer-term, adherence to such diets may still facilitate weight maintenance, for at least up to 12 months (103). In addition, it should be noted that the interpretation of these studies is, in many cases, compromised by poor compliance and high dropout rates, precluding definitive conclusions regarding the long-term effects of high-protein diets on weight loss. However, there is unequivocal evidence that a progressive decrease in adherence is very common with any dietary intervention and, not surprisingly, irrespective of the macronutrient composition, a greater adherence to any energy-restricted diet is associated with a greater weight loss at both one (104) and two years (105). An inherent challenge in longer-term studies is to minimize the impacts of potential cofounders, including the unavoidable lack of blinding, as well as differences in participant characteristics (age, ethnicity and race), which may impact on the GI-induced effects of protein (106, 107).

While there is a lack of definitive evidence regarding the optimal dietary approach for T2D management, in the majority of cases, weight loss represents a primary strategy for improved glycemic control, usually assessed by measurement of glycated hemoglobin (HbA_{1c}). In individuals with prediabetes or newly diagnosed T2D, a modest (5–10%) reduction in body weight improves glycemic control significantly (108). Accordingly, high-protein diets, because of their established weight loss effects, at least in studies of up to 6 months duration, have been advocated as a strategy to improve glycemic control (109). In a recent network meta-analysis of 42 randomized controlled trials, involving 4,809 patients with T2D, comparing the impacts of 10 different dietary approaches on glycemic control, high-protein diets were shown to be among the most effective in reducing both HbA_{1c} and fasting glucose (110). The beneficial effects of high-protein diets to reduce the postprandial blood glucose response, which, in individuals with relatively well-controlled T2D (i.e. baseline HbA_{1c} ≤8.0%), is the major determinant of glycemic control, have been reported in several trials (10, 111–113). However, there are also inconsistent observations, particularly in studies with duration of >6 months (26–28, 114–116). A 2012 meta-analysis, summarizing nine clinical trials with intervention durations between 4 to 24 weeks, revealed a modest, but significant, reduction of 0.52% in $HbA_{1c,}$ but not fasting glucose, in individuals with T2D following a high-protein diet (~25–32% of energy intake) (9). However, no significant effects, on either HbA_{1c} or fasting glucose, were evident in a more recent meta-analysis of 13 trials with intervention durations ranging from 12 weeks to 52 months (116), although, given the large variations in study conditions, this is probably not surprising. Another meta-analysis, which included 15 trials with longer intervention durations (at least 12 months) in individuals with or without T2D, also found no effects on either HbA_{1c} or fasting glucose (28). Therefore, it remains uncertain whether sustained adherence to a high-protein diet improves glycemic control in T2D or prediabetes. It is also not known whether the positive outcomes of shorter-term trials reflect the use of protein per se, the concurrent reduction in weight, or both, particularly since these trials were often based on energy-restricted high-protein diets or incorporated a prior weight-loss period. This issue is, to some extent, semantic given that since 90% of people with T2D are obese in Western countries (117), there is a rationale for high-protein diets as a weight-loss strategy to improve glycemic control. However, whether this represents an effective longer-term approach remains to be established.

A number of studies have investigated the effects of high-protein diets on glycemic variability, which has recently emerged as a target for glycemic control and, potentially, an independent risk factor for the micro- and macrovascular complications of T2D,

particularly when glycemic control is 'reasonable' (i.e. HbA_{1c} ≤8.0%) (113, 118–122). For example, in 16 well-controlled T2D patients, replacing an isocaloric standard-protein (16% protein) with a high-protein (29% protein), diet, for two separate 48-hour periods, was associated with reductions in indices of glycemic variability by 34 to 45%, supporting the concept that a higher intake of protein should be incorporated in dietary advice for patients with T2D (120). Comparable effects were also observed among 20 insulin-resistant women with obesity, where a high-protein diet was more effective in reducing glycemic variability, compared with a Mediterranean diet, in a 21-day trial (121). Furthermore, in a study by Fabricatore et al., in which 26 participants with obesity and T2D underwent a 3-day continuous glucose monitoring (CGM), a higher protein intake was associated with reduced glycemic variability (122). While these findings are promising, confirmation in longer-term studies is required before recommending changes to clinical practice.

A lesser number of studies have examined the effects of selected animal- and plant-based protein sources on weight and glycemia, again with inconsistent outcomes (123–136) (Table 2). While these studies have focused primarily on weight loss, rather than glycemia, observed glucoregulatory effects of different protein sources were in the majority of these studies found not to differ. For example, when the effects of supplemental whey and soy protein (~56 g/d) were compared to an isoenergetic amount of carbohydrate among free-living overweight and obese participants, slightly, but significantly, greater weight loss was observed with whey (-1.8 kg), compared with soy protein (-0.9 kg), with no differences in their effects on fasting glucose (123). In another study of 48 participants with obesity, the effects of two formulas containing either soy (12 g) and milk proteins (9 g) or only milk protein (22 g), given daily every morning for 20 weeks, were compared, and milk protein (-2.5 kg) had superior effects in inducing weight loss than soy protein (-1.1 kg), and also led to a greater reduction in HbA_{1c} levels (124). Another study reported that milk induced a greater reduction in body weight (-4.43 kg) over a period of 8 weeks compared with calcium-fortified soy milk (-3.46 kg) (126). In contrast, greater weight loss effects were reported after 12 weeks with soy- (-9%), rather than milk-based (-7.9%), meal replacements within an energy-restricted diet (125). Interestingly, reductions in fasting glucose were only evident with the soy-, but not with milk-based, meals in this study (125). Consumption of either 3 soy, or 3 casein, shakes daily as part of a 16-week, energy-restricted diet, in two groups of women with obesity, had comparable effects on weight loss and body composition, as well as fasting insulin, while a greater reduction in fasting glucose was evident in the soy group (127). In another study, no difference was found between the weight-reducing effects of a meat-based (-2.2 kg), and a soy-based (-2.4 kg), diet (~30% of energy from protein), with a significant reduction in fasting glucose observed with both diets (130). Two studies reported that beef and chicken, as the primary sources of protein in an energy-restricted diet, had comparable weight loss effects (128, 129). Abete et al. reported that an energy-restricted diet with a high content of legumes (consumed 4 days per week with 17% protein from energy intake) led to body weight reductions comparable to those achieved with a high-protein diet (30% protein) mainly composed of animal proteins, which was associated with significantly greater reduction in fasting glucose only in legume diet group (131). Altogether, there is, therefore, no compelling evidence that a particular protein source leads to greater weight loss, or improvement in glycemia, than another, indicating that plant-based proteins are likely to be as effective for weight loss as animal-based proteins. Moreover, in some cases, plant-based sources were associated with more potent positive glucoregulatory effects (125, 127, 131). Importantly, it remains uncertain whether the effects of the source of protein are independent of other macro- and micronutrient contents.

4 Longer-term effects of dietary protein intake in obesity and T2D

While protein has, for many years, represented the cornerstone of dietary approaches for weight management in obesity, associated with improved glycemic control, there is a lack of consensus regarding the maximal amount of dietary protein that can be consumed in the long-term without adverse effects. Interestingly, in contrast to the beneficial acute and intermediate-term effects of protein on weight loss and glycemic control discussed above, outcomes of large prospective studies investigating the association between the long-term consumption of protein with body weight and/or T2D have indicated no overall beneficial effects (137–139). Moreover, there is evidence that the long-term health effects of protein may vary according to the source of protein, thus, long-term consumption of animal-based proteins may have adverse effects in relation to obesity and T2D, while plant-based proteins have either protective or neutral effects (Figure 2).

Several epidemiological studies, investigating the role of greater protein intake from different sources in the development of obesity in large populations, have consistently reported a direct association between prospective weight gain and higher animal protein intake, and by inference, the risk of obesity (137, 140–142) (Table 3). For example, in the European Prospective Investigation into Cancer and Nutrition (EPIC) study, of 89,432 weight-stable men and women from five countries, overall associations were evident between higher daily intakes of total and animal protein and subsequent weight gain over 6.5 years, which was mainly attributed to protein derived from red and processed meats and chicken, rather than to fish and dairy products (137). In contrast, there were neither protective, nor adverse, associations with plant-based proteins (137). A 2015 analysis, examining the relationships between consumption of different protein sources with long-term weight gain across three separate prospective cohorts of US men and women (the Nurses' Health Studies (NHS) I and II and the Health Professionals Follow-Up Study (HPFS)) revealed that animal-based protein sources were independently associated with long-term weight gain (i.e. each increased serving/day of red meat, chicken and regular cheese was associated with a 0.13–1.17 kg weight gain), whereas plant-based proteins were independently associated with relative weight loss (i.e. each increased serving/day of peanut butter, walnuts or other nuts was associated with

TABLE 2 Intermediate-term effects of different protein sources (animal vs. plant) on energy intake, body weight and glycemia.

First author	Country	Design	Sample size (n)	Age (y)	BMI (kg/m^2)	Health status	Protein source	Protein dose[1]	Duration[2] (week)	EI[3] (kcal/d)	Weight loss[4]	Glycemia[5]	Ref
Baer et al. (2011)	USA	Parallel	48 (M/F)	49-53	31	Overweight and obesity	Soy Whey	56 g/d	23	2,268 2,184	0.9 kg 1.8 kg	0.255* 0.255	(123)
Takahira et al. (2011)	Japan	Parallel	48 (M/F)	54-57	29	Obesity with/ without T2D	Soy Milk	12 g/d SP+9 g/d MP 22 g/d MP	20	1,719 1,799	1.1 kg 2.5 kg	-0.9%** -1.4%	(124)
Anderson et al. (2005)	USA	Parallel	52 (M/F)	46-47	34	Overweight and obesity	Soy Milk	18 g/d 13 g/d	12	NR	9.0%* 7.9%	-3.6%*** -2%	(125)
Faghih et al (2011)	Iran	Parallel	43 (F)	37-38	31	Overweight and obesity	Calcium-fortified soy milk Milk	18% of EI (~57 g/d)	8	1,280 1,297	3.6 kg 4.4 kg	NR	(126)
Anderson et al. (2007)	USA	Parallel	43 (F)	44-46	35	Obesity	Soy Casein	67.2 g/d 62.1 g/d	16	NR	11.9 kg 13.4 kg	5.1 5.2	(127)
Mahon et al. (2007)	USA	Parallel	54 (F)	58	29.6	Obesity with prediabetes	Non-meat protein-based diet Chicken-based diet Beef-based diet	50 g/d 80 g/d 80 g/d	9	1,158 1,098 1,114	5.6 kg 7.9 kg 6.6 kg	5.6 5.6 5.6	(128)
Neacsu et al. (2014)	UKD	Cross-over	20 (M)	51	34.8	Obesity	Soy-based diet Meat-based diet	153 g/d 154 g/d	2	2,072 2,098	2.4 kg 2.2 kg	5.4 5.3	(130)
Abete et al. (2009)	Spain	Parallel	25 (M)	38	31.8	Obesity	Legume-based diet High-protein diet	17% of EI (~74 g/d) 30% of EI (~137 g/d)	8	1,537 1,765	8.3 kg 8.6 kg	-5.1%*** -4.2%	(131)
Melanson et al. (2003)	USA	Parallel	61 (F)	43	32.1	Overweight	Chicken-based diet Beef-based diet	76.3 g/d 72 g/d	12	NR	6 kg 5.6 kg	NR	(129)
Aldrich et al. (2011)	USA	Parallel	12 (M/F)	50	30	Overweight	Mixed protein-diet Whey protein-diet	124 g/d	20	NR	7.6 kg 9.6 kg	NR	(132)

M, Male; F, Female; Y, Years; BMI, Body mass index; EI, Energy intake; T2D, type 2 diabetes; SP, Soy protein; MP, Milk protein; NR, Not reported.
[1] Reported as grams per day intake of protein, otherwise indicated as percentage of daily energy intake (*); [2]Intervention duration; [3] Reported as daily energy intake (kcal); [4]Reported as changes in body weight (kg), otherwise indicated as percentage of weight change (*);
[5] Reported as fasting glucose levels in mmol/L (or log mmol/L*), otherwise indicated as changes in either HbA1c levels% (**) or fasting glucose levels (***).

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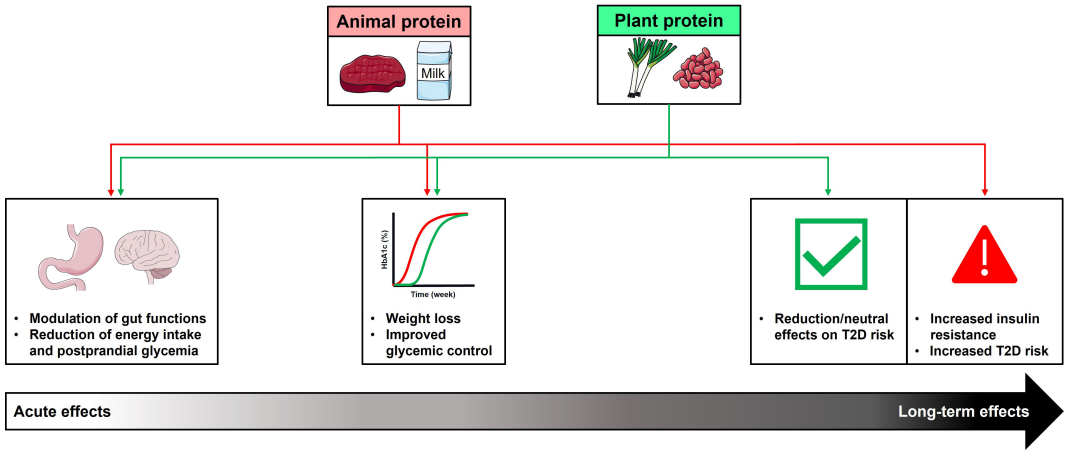

FIGURE 2
Summary of acute, intermediate-, and longer-term effects of dietary animal and plant protein consumption on metabolic health. Acute intakes of protein (animal or plant-based protein), either in an isolated form as 'preloads', or as part of a meal, stimulate GI hormones, associated with reductions in energy intake and postprandial blood glucose. These acute effects are associated with greater weight loss, and improved glycemic control, when consumed as part of a high-protein diet, with comparable outcomes observed with both types of protein. In contrast, the outcomes of longer-term studies suggest that long-term consumption of animal-based protein may have adverse effects in relation to the development of type 2 diabetes (T2D), while plant-based protein have either protective or neutral effects. This may reflect animal protein-specific effects to increase insulin resistance, leading to increased risk of T2D.

-0.14 to -0.71 kg weight loss) over 4 years (140). Similarly, in a recent analysis of the NHS II study, over a 26-year follow-up, intakes of red meat (both fresh and processed products) and high-fat dairy products, were associated with an increased risk of nonalcoholic fatty liver disease (NAFLD), with obesity found to be the major contributor, while a higher intake of nuts was associated with a reduced risk (143). In another cohort of 1,730 employed men, aged 40 to 55 years from the Chicago Western Electric Study, which were followed from 1958 to 1966, animal protein was positively associated with a 4 times greater risk of obesity, while plant protein reduced the risk by 50% (141). Recent studies have also found that substituting different animal protein sources, particularly processed red meats, with plant protein was associated with reduced risks of coronary heart disease (CHD) and all-cause mortality (144–146).

The majority of studies have also reported that long-term consumption of animal protein increased the risk of T2D, while plant proteins had protective or neutral effects (34–36, 147–154) (Table 4). For example, in two large cohort studies (Women's Health Initiative and the UK Biobank), with 16,505 incident cases of T2D (out of 143,297 adults without T2D at baseline), during a median follow-up of 15.8 years, replacing consumption of animal protein (5% of energy intake) with plant protein was associated with a 21% lower risk of T2D, attributable to reductions in obesity-related inflammatory factors (36). Moreover, in another study, a higher intake of animal, but not plant, protein was associated with

TABLE 3 Long-term effects of total, animal and plant protein intake on body weight.

First author	Country	Design	Sample size (n)	Age (y)	BMI (kg/m^2)	Protein source	Protein dose[1]	Duration[2] (y)	Weight change[3]	Ref
Halkjær et al. (2011)	European countries	Prospective cohort	89,432 (M/F)	35-65	20-33	Total protein Animal protein Plant protein	Per increased 150 kcal/d	6.5	+0.052 kg/year (+0.025 to +0.079) +0.056 kg/year (+0.026 to +0.085) +0.017 kg/year (-0.032 to +0.068)	(137)
Bujnowski et al. (2011)	USA	Prospective cohort	1,730 (M)	40-55	24->30	Total protein Animal protein Plant protein	17.2 vs. 13% of EI* 13.8 vs. 9.3% 4.1 vs. 2.9%	7	3.27 (1.94 to 5.51)* 4.62 (2.68 to 7.98) 0.58 (0.36 to 0.95)	(141)
Smith et al. (2015)	USA	Prospective cohort	120,784 (M/F)	30-50	22->25	Animal protein Plant protein	Per increased serving/d	16-24	+0.13 to +1.17 kg/4 years -0.14 to -0.71 kg/4 years	(140)

M, Male; F, Female; Y, Years; BMI, Body mass index; EI, Energy intake.
[1] Reported as grams per day intake of protein, otherwise indicated as percentage of daily energy intake (*); [2] Follow up duration; [3] Reported as either body weight change (kg), otherwise indicated as risk of obesity (*).

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TABLE 4 Long-term effects of total, animal and plant protein intake on glycemic control.

First author	Country	Design	Sample size (n)	Age (y)	BMI (kg/m^2)	Protein source	Protein dose[1]	Duration[2] (y)	T2D cases (n)	T2D risk	Ref
Song et al. (2004)	USA	Prospective cohort	37,309 (F)	>45	20->30	Animal protein Plant protein	77 vs. 40 g/d 37 vs. 17 g/d	8.8	1,558	1.44 (1.16 to 1.78) 0.85 (0.70 to 1.03)	(150)
Sluijs et al. (2010)	The Netherlands	Prospective cohort	38,094 (M/F)	21-70	24->30	Total protein Animal protein Plant protein	Per increased 10 g/d	10.1	918	1.16 (1.06 to 1.26) 1.13 (1.04 to 1.22) 1.04 (0.83 to 1.29)	(147)
van Nielen et al. (2014)	European countries	Prospective cohort	26,253 (M/F)	53 (mean)	<25->30	Total protein Animal protein Plant protein	Per 10 g/d	12	11,637	1.06 (1.02 to 1.09) 1.05 (1.02 to 1.08) 1.04 (0.93 to 1.16)	(152)
Malik et al. (2016)	USA	Prospective cohort	205,802 (M/F)	30-50	22->25	Total protein Animal protein Plant protein	22 vs. 14% of EI* 17 vs. 9% 7 vs. 4%	20.1	15,580	1.07 (1.01 to 1.17) 1.13 (1.06 to 1.21) 0.91 (0.84 to 0.98)	(34)
Shang et al. (2016)	AUS	Prospective cohort	21,523 (M/F)	27-80	20->30	Total protein Animal protein Plant protein	Per 5% of EI*	11.7	929	1.15 (1.00 to 1.32) 1.15 (1.00 to 1.33) 1.00 (0.69 to 1.46)	(149)
Sugihiro et al. (2019)	USA	Prospective cohort	765 (M/F)	58.1 (mean)	≥25	Total protein Animal protein Plant protein	Per 1% of EI*	10.7	36	1.22 (1.03 to 1.45) 1.20 (1.04 to 1.38) 0.82 (0.62 to 1.09)	(151)
Chen et al. (2020)	The Netherlands	Prospective cohort	6,813 (M/F)	≥45	–	Total protein Animal protein Plant protein	Per 5% of EI*	7.2	643	1.37 (1.18 to 1.58) 1.37 (1.19 to 1.58) 1.21 (0.83 to 1.77)	(154)
Yuan et al. (2021)	China	Prospective cohort	7,312 (M/F)	48.3 (mean)	22->25	Total protein Animal protein Plant protein	92 vs. 41 g/d 41 vs. 3 g/d 65 vs. 25 g/d	5.8	209	2.38 (1.43 to 3.98) 1.93 (1.17 to 3.17) 1.20 (0.71 to 2.04)	(153)
Li et al. (2022)	USA UKD	Prospective cohort	108,681 (F) 34,616 (F)	50-80	22->30	Total protein Animal protein Plant protein Total protein	86 vs. 50 g/d 67 vs. 28 g/d 28 vs. 13 g/d Per 5% of EI*	15.8 11.4	15,842 663	1.24 (1.18 to 1.30) 1.31 (1.24 to 1.37) 0.82 (0.78 to 0.86) 1.14 (0.99-1.32)	(36)

M, Male; F, Female; Y, Years; BMI, Body mass index; T2D, Type 2 diabetes; EI, energy intake.
[1] Reported as grams per day intake of protein, otherwise indicated as percentage of daily energy intake (*); [2] Follow up duration.

increased risks of both prediabetes and T2D; so that each 5% increment in energy intake from animal protein at the expense of carbohydrate was associated with increased risks of prediabetes of 35% and T2D of 37% (154). This was attributable primarily to increased insulin resistance, as assessed by the indirect homeostatic model (HOMA-IR) (154). A number of recent reviews and meta-analyses have also concluded that higher animal, but not plant, protein intake is associated with an increased risk of T2D (155–160).

The mechanisms through which a high protein intake, from animal vs. plant-based sources, may have differential impacts in the long-term are poorly defined. However, there are several potential explanations, including differences in amino acid composition, glycemic load and potential deleterious effects of the high insulinotropic properties of animal protein, which, in turn, promotes fat storage and impedes fat oxidation (161, 162). Preclinical models also indicate that increased levels of specific amino acids, particularly BCAAs, which are abundant in animal-based proteins, could lead to insulin resistance by activating mTOR, to initiate a detrimental feedback loop toward insulin receptor substrate 1 signaling (163, 164). Indeed, insulin-mediated glucose uptake decreases when body tissues are chronically overexposed to high levels of insulin. Thus, prolonged hyperinsulinemia may lead to insulin resistance and, ultimately, T2D. Elevated postprandial levels of BCAAs have also been shown to inhibit muscle glucose transport and/or glucose phosphorylation directly, to reduce glycogen synthesis, further contributing to insulin resistance (165). Limited human studies also indicate that an increase in protein intake in the longer-term can reduce insulin sensitivity (166, 167). For example, in healthy participants, a higher consumption of protein (~1.87 g/kg of body weight) for six months was associated with greater glucose-stimulated insulin secretion and a modest reduction in insulin sensitivity (166). In another study, in overweight participants, comparing an isoenergetic high-protein diet (~25–30% protein) with a conventional-protein (~15% protein) control diet over 18 weeks, a reduction in insulin sensitivity, as measured by the euglycemic hyper-insulinemic clamp, was observed (167). The differential impacts of animal and plant protein may also be influenced by other dietary nutrients. For example, plant-based foods are rich in dietary fiber, which is known to mitigate T2D risk and may interact additively with plant protein (168). In contrast, a number of dietary components in red and processed meats, as the primary sources of animal protein, such as heme iron, animal fat, and advanced glycation end products, may be, both directly and indirectly, associated with an increased T2D risk. This association may reflect factors including obesity and its related inflammatory markers (leptin and endothelial dysfunction biomarkers) (36, 154, 169).

It is important to also appreciate other potential deleterious effects of high-protein diets, particularly increased risks of osteoporosis and renal diseases (170–174). A potential link with osteoporosis was supported by the observation of increased urinary calcium excretion during a high protein intake (170–172). High-protein diets (>2 g/kg/day) may also increase bone resorption by increasing the acid load in the body, compared with diets of low- to normal-protein content of 0.7-1.0 g/kg/day (172). Indeed, it has been suggested that high consumption of animal-based protein, in particular, leads to an acidification of the blood that may increase carbonate, and subsequently calcium, release from the skeleton to decrease bone mineral density (171). In an epidemiological study of older men (>60 years), a greater dietary acid load due to a chronic high-protein intake was associated with femoral bone loss only under conditions of very low calcium intake <800 mg/d dietary calcium (173). An increased renal acid load, such as the sulfuric acid produced from the oxidation of different amino acids, has also been suggested to increase the risk of kidney stones, and/or increase the glomerular filtration rate, which may lead to renal dysfunction over time (174). While these findings are yet to be confirmed in different populations, they further support that recommendations for a higher protein intake in the long-term should be circumspect.

5 Conclusions and recommendations/ priorities for future studies

There is strong evidence from short-term studies (i.e. <6 months in duration) that a higher dietary protein intake facilitates weight loss in obesity and improves glycemic control in T2D. In contrast, the outcomes of longer-term studies, of which there are less, are not convincing, precluding clear-cut recommendations. Suboptimal dietary adherence and metabolic adaptations are likely to contribute to this apparent anomaly, as well as methodological limitations with respect to the type and duration of studies, characteristics of study participants, and how well-controlled the studies are. While acute studies are well-controlled and provide the most reliable findings, these are characteristically performed among a smaller number of participants, who are predominantly young. Accordingly, longer-term studies with larger and more heterogeneous populations are required. An important issue, which has received inappropriately little attention, is the source of dietary protein (i.e. animal vs. plant). The importance of this issue is highlighted by recent epidemiological studies, which strongly support the concept that animal-, but not plant-, based protein intake may have adverse effects in relation to the development of obesity and T2D. Importantly, the longer-term comparative effects of high-protein diets, based on different sources, on body weight and glycemic control remain to be formally evaluated. Despite this limitation, it would be appropriate for current dietary guidelines to consider the source of dietary protein in relation to the use of high-protein diets, and reasonable to advise a reduction in the consumption of animal protein and a relatively increased intake of plant protein. Such a nuanced approach may prove fundamental to longer-term outcomes. Moreover, future studies should focus on the relevance of animal vs. plant-based protein sources, particularly how longer-term consumption of different protein sources may affect GI-related food intake- and gluco-regulatory mechanisms. The outcomes of such studies are likely to lead to more personalized and effective use of protein in the prevention and management of obesity and T2D.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Leidy HJ, Clifton PM, Astrup A, Wycherley TP, Westerterp-Plantenga MS, Luscombe-Marsh ND, et al. The role of protein in weight loss and maintenance. *Am J Clin Nutr*. (2015) 101:1320S–9S. doi: 10.3945/ajcn.114.084038

2. Halton TL, Hu FB. The effects of high protein diets on thermogenesis, satiety and weight loss: a critical review. *J Am Coll Nutr*. (2004) 23:373–85. doi: 10.1080/07315724.2004.10719381

3. Astrup A, Raben A, Geiker N. The role of higher protein diets in weight control and obesity-related comorbidities. *Int J Obes (Lond)*. (2015) 39:721–6. doi: 10.1038/ijo.2014.216

4. Westerterp-Plantenga MS, Nieuwenhuizen A, Tomé D, Soenen S, Westerterp KR. Dietary protein, weight loss, and weight maintenance. *Annu Rev Nutr*. (2009) 29:21–41. doi: 10.1146/annurev-nutr-080508-141056

5. Te Morenga L, Mann J. The role of high-protein diets in body weight management and health. *Br J Nutr*. (2012) 108:130–8. doi: 10.1017/S0007114512002437

6. League Of Nations. Report on the physiological bases of nutrition drawn up by the Technical Commission of the Health Committee. *Problem Nutr*. (1936).

7. Joint WHO/FAO/UNU expert consultation. Protein and amino acid requirements in human nutrition. *World Health Organ Tech Rep Ser*. (2007) 935:1–265.

8. Wycherley TP, Moran LJ, Clifton PM, Noakes M, Brinkworth GD. Effects of energy-restricted high-protein, low-fat compared with standard-protein, low-fat diets: a meta-analysis of randomized controlled trials. *Am J Clin Nutr*. (2012) 96:1281–98. doi: 10.3945/ajcn.112.044321

9. Dong J-Y, Zhang Z-L, Wang P-Y, Qin L-Q. Effects of high-protein diets on body weight, glycaemic control, blood lipids and blood pressure in type 2 diabetes: meta-analysis of randomised controlled trials. *Br J Nutr*. (2013) 110:781–9. doi: 10.1017/S0007114513002055

10. Gannon MC, Nuttall FQ, Saeed A, Jordan K, Hoover H. An increase in dietary protein improves the blood glucose response in persons with type 2 diabetes. *Am J Clin Nutr*. (2003) 78:734–41. doi: 10.1093/ajcn/78.4.734

11. Bowen J, Noakes M, Trenerry C, Clifton PM. Energy intake, ghrelin, and cholecystokinin after different carbohydrate and protein preloads in overweight men. *J Clin Endocrinol Metab*. (2006) 91:1477–83. doi: 10.1210/jc.2005-1856

12. Brennan IM, Luscombe-Marsh ND, Seimon RV, Otto B, Horowitz M, Wishart JM, et al. Effects of fat, protein, and carbohydrate and protein load on appetite, plasma cholecystokinin, peptide YY, and ghrelin, and energy intake in lean and obese men. *Am J Physiol Gastrointest Liver Physiol*. (2012) 303:129–40. doi: 10.1152/ajpgi.00478.2011

13. Hutchison AT, Piscitelli D, Horowitz M, Jones KL, Clifton PM, Standfield S, et al. Acute load-dependent effects of oral whey protein on gastric emptying, gut hormone release, glycemia, appetite, and energy intake in healthy men. *Am J Clin Nutr*. (2015) 102:1574–84. doi: 10.3945/ajcn.115.117556

14. Akhavan T, Luhovyy BL, Brown PH, Cho CE, Anderson GH. Effect of premeal consumption of whey protein and its hydrolysate on food intake and postmeal glycemia and insulin responses in young adults. *Am J Clin Nutr*. (2010) 91:966–75. doi: 10.3945/ajcn.2009.28406

15. Astbury NM, Stevenson EJ, Morris P, Taylor MA, Macdonald IA. Dose–response effect of a whey protein preload on within-day energy intake in lean subjects. *Br J Nutr*. (2010) 104:1858–67. doi: 10.1017/S000711451000293X

16. Bertenshaw EJ, Lluch A, Yeomans MR. Dose-dependent effects of beverage protein content upon short-term intake. *Appetite*. (2009) 52:580–7. doi: 10.1016/j.appet.2009.01.010

17. Ma J, Stevens JE, Cukier K, Maddox AF, Wishart JM, Jones KL, et al. Effects of a protein preload on gastric emptying, glycemia, and gut hormones after a carbohydrate meal in diet-controlled type 2 diabetes. *Diabetes Care*. (2009) 32:1600–2. doi: 10.2337/dc09-0723

18. Ma J, Jesudason DR, Stevens JE, Keogh JB, Jones KL, Clifton PM, et al. Sustained effects of a protein 'preload' on glycaemia and gastric emptying over 4 weeks in patients with type 2 diabetes: A randomized clinical trial. *Diabetes Res Clin Pract*. (2015) 108:31–4. doi: 10.1016/j.diabres.2015.02.019

19. Nuttall FQ, Mooradian AD, Gannon MC, Billington C, Krezowski P. Effect of protein ingestion on the glucose and insulin response to a standardized oral glucose load. *Diabetes Care*. (1984) 7:465–70. doi: 10.2337/diacare.7.5.465

20. Belza A, Ritz C, Sørensen MQ, Holst JJ, Rehfeld JF, Astrup A. Contribution of gastroenteropancreatic appetite hormones to protein-induced satiety. *Am J Clin Nutr*. (2013) 97:980–9. doi: 10.3945/ajcn.112.047563

21. Fromentin G, Darcel N, Chaumontet C, Marsset-Baglieri A, Nadkarni N, Tomé D. Peripheral and central mechanisms involved in the control of food intake by dietary amino acids and proteins. *Nutr Res Rev*. (2012) 25:29–39. doi: 10.1017/S0954422411000175

22. Woods SC, D'Alessio DA. Central control of body weight and appetite. *J Clin Endocrinol Metab*. (2008) 93:37–50. doi: 10.1210/jc.2008-1630

23. Steinert RE, Feinle-Bisset C, Asarian L, Horowitz M, Beglinger C, Geary N. Ghrelin, CCK, GLP-1, and PYY(3-36): secretory controls and physiological roles in eating and glycemia in health, obesity, and after RYGB. *Physiol Rev*. (2017) 97:411–63. doi: 10.1152/physrev.00031.2014

24. Camilleri M. Peripheral mechanisms in appetite regulation. *Gastroenterology*. (2015) 148:1219–33. doi: 10.1053/j.gastro.2014.09.016

25. Marathe CS, Rayner CK, Jones KL, Horowitz M. Relationships between gastric emptying, postprandial glycemia, and incretin hormones. *Diabetes Care*. (2013) 36:1396–405. doi: 10.2337/dc12-1609

26. Brinkworth G, Noakes M, Keogh J, Luscombe N, Wittert G, Clifton P. Long-term effects of a high-protein, low-carbohydrate diet on weight control and cardiovascular risk markers in obese hyperinsulinemic subjects. *Int J Obes Relat Metab Disord*. (2004) 28:661–70. doi: 10.1038/sj.ijo.0802617

27. Brinkworth G, Noakes M, Parker B, Foster P, Clifton P. Long-term effects of advice to consume a high-protein, low-fat diet, rather than a conventional weight-loss

diet, in obese adults with type 2 diabetes: one-year follow-up of a randomised trial. *Diabetologia*. (2004) 47:1677–86. doi: 10.1007/s00125-004-1511-7

28. Schwingshackl L, Hoffmann G. Long-term effects of low-fat diets either low or high in protein on cardiovascular and metabolic risk factors: a systematic review and meta-analysis. *Nutr J*. (2013) 12:48. doi: 10.1186/1475-2891-12-48

29. Pasiakos SM, Agarwal S, Lieberman HR, Fulgoni VLIII. Sources and amounts of animal, dairy, and plant protein intake of US adults in 2007–2010. *Nutrients*. (2015) 7:7058–69. doi: 10.3390/nu7085322

30. Miller V, Reedy J, Cudhea F, Zhang J, Shi P, Erndt-Marino J, et al. Global, regional, and national consumption of animal-source foods between 1990 and 2018: findings from the Global Dietary Database. *Lancet Planet Health*. (2022) 6:243–56. doi: 10.1016/S2542-5196(21)00352-1

31. Naghshi S, Sadeghi O, Willett WC, Esmaillzadeh A. Dietary intake of total, animal, and plant proteins and risk of all cause, cardiovascular, and cancer mortality: systematic review and dose-response meta-analysis of prospective cohort studies. *BMJ*. (2020) 370:2412. doi: 10.1136/bmj.m2412

32. Neuenschwander M, Stadelmaier J, Eble J, Grummich K, Szczerba E, Kiesswetter E, et al. Substitution of animal-based with plant-based foods on cardiometabolic health and all-cause mortality: a systematic review and meta-analysis of prospective studies. *BMC Med*. (2023) 21:404. doi: 10.1186/s12916-023-03093-1

33. Ferrari L, Panaite S-A, Bertazzo A, Visioli F. Animal- and plant-based protein sources: a scoping review of human health outcomes and environmental impact. *Nutrients*. (2022) 14:5115. doi: 10.3390/nu14235115

34. Malik VS, Li Y, Tobias DK, Pan A, Hu FB. Dietary protein intake and risk of type 2 diabetes in us men and women. *Am J Epidemiol*. (2016) 183:715–28. doi: 10.1093/aje/kwv268

35. Ericson U, Sonestedt E, Gullberg B, Hellstrand S, Hindy G, Wirfält E, et al. High intakes of protein and processed meat associate with increased incidence of type 2 diabetes. *Br J Nutr*. (2013) 109:1143–53. doi: 10.1017/S0007114512003017

36. Li J, Glenn AJ, Yang Q, Ding D, Zheng L, Bao W, et al. Dietary protein sources, mediating biomarkers, and incidence of type 2 diabetes: findings from the Women's Health Initiative and the UK Biobank. *Diabetes Care*. (2022) 45:1742–53. doi: 10.2337/dc22-0368

37. Burton-Freeman BM. Glycomacropeptide (GMP) is not critical to whey-induced satiety, but may have a unique role in energy intake regulation through cholecystokinin (CCK). *Physiol Behav*. (2008) 93:379–87. doi: 10.1016/j.physbeh.2007.09.010

38. Kohanmoo A, Faghih S, Akhlaghi M. Effect of short- and long-term protein consumption on appetite and appetite-regulating gastrointestinal hormones, a systematic review and meta-analysis of randomized controlled trials. *Physiol Behav*. (2020) 226:113123. doi: 10.1016/j.physbeh.2020.113123

39. Smedegaard S, Kampmann U, Ovesen PG, Støvring H, Rittig N. Whey protein premeal lowers postprandial glucose concentrations in adults compared with water - the effect of timing, dose, and metabolic status: a systematic review and meta-analysis. *Am J Clin Nutr*. (2023) 118:391–405. doi: 10.1016/j.ajcnut.2023.05.012

40. Gheldof N, Francey C, Rytz A, Egli L, Delodder F, Bovetto L, et al. Effect of different nutritional supplements on glucose response of complete meals in two crossover studies. *Nutrients*. (2022) 14:2674. doi: 10.3390/nu14132674

41. Gordon RA, Zumbro EL, Castleberry TJ, Sokoloski ML, Brisebois MF, Irvine CJ, et al. Whey protein improves glycemia during an oral glucose tolerance test compared to vigorous-intensity aerobic exercise in young adult men. *BMC Sports Sci Med Rehabil*. (2022) 14:147. doi: 10.1186/s13102-022-00540-z

42. Smith HA, Watkins JD, Walhin JP, Gonzalez JT, Thompson D, Betts JA. Whey protein-enriched and carbohydrate-rich breakfasts attenuate insulinemic responses to an ad libitum lunch relative to extended morning fasting: a randomized crossover trial. *J Nutr*. (2023) 153:2842–53. doi: 10.1016/j.tjnut.2023.08.008

43. Acheson KJ, Blondel-Lubrano A, Oguey-Araymon S, Beaumont M, Emady-Azar S, Ammon-Zufferey C, et al. Protein choices targeting thermogenesis and metabolism. *Am J Clin Nutr*. (2011) 93:525–34. doi: 10.3945/ajcn.110.005850

44. Anderson GH, Tecimer SN, Shah D, Zafar TA. Protein source, quantity, and time of consumption determine the effect of proteins on short-term food intake in young men. *J Nutr*. (2004) 134:3011–5. doi: 10.1093/jn/134.11.3011

45. Bowen J, Noakes M, Clifton PM. Appetite regulatory hormone responses to various dietary proteins differ by body mass index status despite similar reductions in ad libitum energy intake. *J Clin Endocrinol Metab*. (2006) 91:2913–9. doi: 10.1210/jc.2006-0609

46. Diepvens K, Häberer D, Westerterp-Plantenga M. Different proteins and biopeptides differently affect satiety and anorexigenic/orexigenic hormones in healthy humans. *Int J Obes (Lond)*. (2008) 32:510–8. doi: 10.1038/sj.ijo.0803758

47. Dougkas A, Östman E. Comparable effects of breakfast meals varying in protein source on appetite and subsequent energy intake in healthy males. *Eur J Nutr*. (2018) 57:1097–108. doi: 10.1007/s00394-017-1392-4

48. Gunnerud UJ, Heinzle C, Holst JJ, Östman EM, Björck IM. Effects of pre-meal drinks with protein and amino acids on glycemic and metabolic responses at a subsequent composite meal. *PloS One*. (2012) 7:44731. doi: 10.1371/journal.pone.0044731

49. Hall W, Millward D, Long S, Morgan L. Casein and whey exert different effects on plasma amino acid profiles, gastrointestinal hormone secretion and appetite. *Br J Nutr*. (2003) 89:239–48. doi: 10.1079/BJN2002760

50. Hoefle AS, Bangert AM, Stamfort A, Gedrich K, Rist MJ, Lee Y-M, et al. Metabolic responses of healthy or prediabetic adults to bovine whey protein and sodium caseinate do not differ. *J Nutr*. (2015) 145:467–75. doi: 10.3945/jn.114.199190

51. Melson CE, Nepocatych S, Madzima TA. The effects of whey and soy liquid breakfast on appetite response, energy metabolism, and subsequent energy intake. *Nutrition*. (2019) 61:179–86. doi: 10.1016/j.nut.2018.11.007

52. Mortensen LS, Hartvigsen ML, Brader LJ, Astrup A, Schrezenmeir J, Holst JJ, et al. Differential effects of protein quality on postprandial lipemia in response to a fat-rich meal in type 2 diabetes: comparison of whey, casein, gluten, and cod protein. *Am J Clin Nutr*. (2009) 90:41–8. doi: 10.3945/ajcn.2008.27281

53. Pal S, Ellis V. The acute effects of four protein meals on insulin, glucose, appetite and energy intake in lean men. *Br J Nutr*. (2010) 104:1241–8. doi: 10.1017/S0007114510001911

54. Teunissen-Beekman KF, Dopheide J, Geleijnse JM, Bakker SJ, Brink EJ, de Leeuw PW, et al. Differential effects of proteins and carbohydrates on postprandial blood pressure-related responses. *Br J Nutr*. (2014) 112:600–8. doi: 10.1017/S0007114514001251

55. Veldhorst MA, Nieuwenhuizen AG, Hochstenbach-Waelen A, Westerterp KR, Engelen MP, Brummer R-JM, et al. A breakfast with alpha-lactalbumin, gelatin, or gelatin+ TRP lowers energy intake at lunch compared with a breakfast with casein, soy, whey, or whey-GMP. *Clin Nutr*. (2009) 28:147–55. doi: 10.1016/j.clnu.2008.12.003

56. Veldhorst MAB, Nieuwenhuizen AG, Hochstenbach-Waelen A, van Vught AJAH, Westerterp KR, Engelen MPKJ, et al. Dose-dependent satiating effect of whey relative to casein or soy. *Physiol Behav*. (2009) 96:675–82. doi: 10.1016/j.physbeh.2009.01.004

57. Nilsson M, Stenberg M, Frid AH, Holst JJ, Björck IME. Glycemia and insulinemia in healthy subjects after lactose-equivalent meals of milk and other food proteins: the role of plasma amino acids and incretins. *Am J Clin Nutr*. (2004) 80:1246–53. doi: 10.1093/ajcn/80.5.1246

58. Pham T, Knowles S, Bermingham E, Brown J, Hannaford R, Cameron-Smith D, et al. Plasma amino acid appearance and status of appetite following a single meal of red meat or a plant-based meat analog: a randomized crossover clinical trial. *Curr Dev Nutr*. (2022) 6:nzac082. doi: 10.1093/cdn/nzac082

59. Sambashivaiah S, Cope M, Mukherjea R, Selvam S, George N, Kuriyan R, et al. The Effect of soy and whey protein supplementation on glucose homeostasis in healthy normal weight Asian Indians. *J Nutr Metab*. (2023) 2023:2622057. doi: 10.1155/2023/2622057

60. Boirie Y, Dangin M, Gachon P, Vasson MP, Maubois JL, Beaufrère B. Slow and fast dietary proteins differently modulate postprandial protein accretion. *Proc Natl Acad Sci U.S.A*. (1997) 94:14930–5. doi: 10.1073/pnas.94.26.14930

61. Ryan AT, Feinle-Bisset C, Kallas A, Wishart JM, Clifton PM, Horowitz M, et al. Intraduodenal protein modulates antropyloroduodenal motility, hormone release, glycemia, appetite, and energy intake in lean men. *Am J Clin Nutr*. (2012) 96:474–82. doi: 10.3945/ajcn.112.038133

62. Hutchison AT, Feinle-Bisset C, Fitzgerald PC, Standfield S, Horowitz M, Clifton PM, et al. Comparative effects of intraduodenal whey protein hydrolysate on antropyloroduodenal motility, gut hormones, glycemia, appetite, and energy intake in lean and obese men. *Am J Clin Nutr*. (2015) 102:1323–31. doi: 10.3945/ajcn.115.114538

63. Steinert RE, Landrock MF, Ullrich SS, Standfield S, Otto B, Horowitz M, et al. Effects of intraduodenal infusion of the branched-chain amino acid leucine on ad libitum eating, gut motor and hormone functions, and glycemia in healthy men. *Am J Clin Nutr*. (2015) 102:820–7. doi: 10.3945/ajcn.115.114488

64. Steinert RE, Luscombe-Marsh ND, Little TJ, Standfield S, Otto B, Horowitz M, et al. Effects of intraduodenal infusion of l-tryptophan on ad libitum eating, antropyloroduodenal motility, glycemia, insulinemia, and gut peptide secretion in healthy men. *J Clin Endocrinol Metab*. (2014) 99:3275–84. doi: 10.1210/jc.2014-1943

65. Hajishafiee M, Ullrich SS, Fitzgerald PC, Horowitz M, Lange K, Poppitt SD, et al. Suppression of energy intake by intragastric l-tryptophan in lean and obese men: relations with appetite perceptions and circulating cholecystokinin and tryptophan. *J Nutr*. (2021) 151:2932–41. doi: 10.1093/jn/nxab218

66. Ullrich SS, Fitzgerald PCE, Giesbertz P, Steinert RE, Horowitz M, Feinle-Bisset C. Effects of intragastric administration of tryptophan on the blood glucose response to a nutrient drink and energy intake, in lean and obese men. *Nutrients*. (2018) 10:463. doi: 10.3390/nu10040463

67. Elovaris RA, Bitarafan V, Agah S, Ullrich SS, Lange K, Horowitz M, et al. Comparative effects of the branched-chain amino acids, leucine, isoleucine and valine, on gastric emptying, plasma glucose, c-peptide and glucagon in healthy men. *Nutrients*. (2021) 13:1613. doi: 10.3390/nu13051613

68. Elovaris RA, Hajishafiee M, Ullrich SS, Fitzgerald PCE, Lange K, Horowitz M, et al. Intragastric administration of leucine and isoleucine does not reduce the glycaemic response to, or slow gastric emptying of, a carbohydrate-containing drink in type 2 diabetes. *Diabetes Res Clin Pract*. (2021) 171:108618. doi: 10.1016/j.diabres.2020.108618

69. Fitzgerald PCE, Manoliu B, Herbillon B, Steinert RE, Horowitz M, Feinle-Bisset C. Effects of L-phenylalanine on energy intake and glycaemia-impacts on appetite perceptions, gastrointestinal hormones and gastric emptying in healthy males. *Nutrients*. (2020) 12:1788. doi: 10.3390/nu12061788

70. Steinert RE, Ullrich SS, Geary N, Asarian L, Bueter M, Horowitz M, et al. Comparative effects of intraduodenal amino acid infusions on food intake and gut hormone release in healthy males. *Physiol Rep*. (2017) 5:13492. doi: 10.14814/phy2.13492

71. de Lartigue G, Diepenbroek C. Novel developments in vagal afferent nutrient sensing and its role in energy homeostasis. *Curr Opin Pharmacol*. (2016) 31:38–43. doi: 10.1016/j.coph.2016.08.007

72. Furness JB, Rivera LR, Cho H-J, Bravo DM, Callaghan B. The gut as a sensory organ. *Nat Rev Gastroenterol Hepatol*. (2013) 10:729–40. doi: 10.1038/nrgastro.2013.180

73. Horowitz M, Edelbroek M, Wishart J, Straathof J. Relationship between oral glucose tolerance and gastric emptying in normal healthy subjects. *Diabetologia*. (1993) 36:857–62. doi: 10.1007/BF00400362

74. Jalleh RJ, Wu T, Jones KL, Rayner CK, Horowitz M, Marathe CS. Relationships of glucose, GLP-1, and insulin secretion with gastric emptying after a 75-g glucose load in type 2 diabetes. *J Clin Endocrinol Metab*. (2022) 107:3850–6. doi: 10.1210/clinem/dgac330

75. Young A. Inhibition of glucagon secretion. *Adv Pharmacol*. (2005) 52:151–71. doi: 10.1016/S1054-3589(05)52008-8

76. Ahmed M, Nuttall FQ, Gannon MC, Lamusga RF. Plasma glucagon and alpha-amino acid nitrogen response to various diets in normal humans. *Am J Clin Nutr*. (1980) 33:1917–24. doi: 10.1093/ajcn/33.9.1917

77. Watson LE, Phillips LK, Wu T, Bound MJ, Checklin H, Grivell J, et al. Differentiating the effects of whey protein and guar gum preloads on postprandial glycemia in type 2 diabetes. *Clin Nutr*. (2019) 38:2827–32. doi: 10.1016/j.clnu.2018

78. Watson LE, Phillips LK, Wu T, Bound MJ, Checklin HL, Grivell J, et al. A whey/guar "preload" improves postprandial glycaemia and glycated haemoglobin levels in type 2 diabetes: A 12-week, single-blind, randomized, placebo-controlled trial. *Diabetes Obes Metab*. (2019) 21:930–8. doi: 10.1111/dom.13604

79. Elovaris RA, Hutchison AT, Lange K, Horowitz M, Feinle-Bisset C, Luscombe-Marsh ND. Plasma free amino acid responses to whey protein and their relationships with gastric emptying, blood glucose- and appetite-regulatory hormones and energy intake in lean healthy men. *Nutrients*. (2019) 11:2465. doi: 10.3390/nu11102465

80. Luscombe-Marsh ND, Hutchison AT, Soenen S, Steinert RE, Clifton PM, Horowitz M, et al. Plasma free amino acid responses to intraduodenal whey protein, and relationships with insulin, glucagon-like peptide-1 and energy intake in lean healthy men. *Nutrients*. (2016) 8:4. doi: 10.3390/nu8010004

81. Nuttall FQ, Gannon MC, Jordan K. The metabolic response to ingestion of proline with and without glucose. *Metabolism*. (2004) 53:241–6. doi: 10.1016/j.metabol.2003.09.013

82. Kalogeropoulou D, LaFave L, Schweim K, Gannon MC, Nuttall FQ. Lysine ingestion markedly attenuates the glucose response to ingested glucose without a change in insulin response. *Am J Clin Nutr*. (2009) 90:314–20. doi: 10.3945/ajcn.2008.27381

83. Anjom-Shoae J, Fitzgerald PCE, Horowitz M, Mohammadpour Z, Gv H, JJ H, et al. Intraduodenal calcium enhances the effects of L-tryptophan to stimulate gut hormone secretion and suppress energy intake in healthy males: a randomized, cross-over, clinical trial. *Am J Clin Nutr*. (2024) S0002-9165:00602–6. doi: 10.1016/j.ajcnut.2024.07.006

84. Mellinkoff SM, Frankland M, Boyle D, Greipel M. Relationship between serum amino acid concentration and fluctuations in appetite. *1956. Obes Res*. (1997) 5:381–4. doi: 10.1002/j.1550-8528.1997.tb00568.x

85. Takahara T, Amemiya Y, Sugiyama R, Maki M, Shibata H. Amino acid-dependent control of mTORC1 signaling: a variety of regulatory modes. *J BioMed Sci*. (2020) 27:87. doi: 10.1186/s12929-020-00679-2

86. Meijer AJ, Dubbelhuis PF. Amino acid signalling and the integration of metabolism. *Biochem Biophys Res Commun*. (2004) 313:397–403. doi: 10.1016/j.bbrc.2003.07.012

87. Blandino-Rosano M, Chen AY, Scheys JO, Alejandro EU, Gould AP, Taranukha T, et al. mTORC1 signaling and regulation of pancreatic β-cell mass. *Cell Cycle*. (2012) 11:1892–902. doi: 10.4161/cc.20036

88. Yoon MS. The emerging role of branched-chain amino acids in insulin resistance and metabolism. *Nutrients*. (2016) 8:405. doi: 10.3390/nu8070405

89. Berger M, Gray JA, Roth BL. The expanded biology of serotonin. *Annu Rev Med*. (2009) 60:355–66. doi: 10.1146/annurev.med.60.042307.110802

90. He W, Wu G. Metabolism of amino acids in the brain and their roles in regulating food intake. *Adv Exp Med Biol*. (2020) 1265:167–85. doi: 10.1007/978-3-030-45328-2_10

91. Wellman PJ. Modulation of eating by central catecholamine systems. *Curr Drug Targets*. (2005) 6:191–9. doi: 10.2174/1389450053174532

92. Skov A, Toubro S, Rønn B, Holm L, Astrup A. Randomized trial on protein vs carbohydrate in ad libitum fat reduced diet for the treatment of obesity. *Int J Obes Relat Metab Disord*. (1999) 23:528–36. doi: 10.1038/sj.ijo.0800867

93. McAuley K, Hopkins C, Smith K, McLay R, Williams S, Taylor R, et al. Comparison of high-fat and high-protein diets with a high-carbohydrate diet in insulin-resistant obese women. *Diabetologia*. (2005) 48:8–16. doi: 10.1007/s00125-004-1603-4

94. Claessens M, Van Baak M, Monsheimer S, Saris W. The effect of a low-fat, high-protein or high-carbohydrate ad libitum diet on weight loss maintenance and metabolic risk factors. *Int J Obes (Lond)*. (2009) 33:296–304. doi: 10.1038/ijo.2008.278

95. Farnsworth E, Luscombe ND, Noakes M, Wittert G, Argyiou E, Clifton PM. Effect of a high-protein, energy-restricted diet on body composition, glycemic control, and lipid concentrations in overweight and obese hyperinsulinemic men and women. *Am J Clin Nutr*. (2003) 78:31–9. doi: 10.1093/ajcn/78.1.31

96. Layman DK, Boileau RA, Erickson DJ, Painter JE, Shiue H, Sather C, et al. A reduced ratio of dietary carbohydrate to protein improves body composition and blood lipid profiles during weight loss in adult women. *J Nutr*. (2003) 133:411–7. doi: 10.1093/jn/133.2.411

97. Santesso N, Akl EA, Bianchi M, Mente A, Mustafa R, Heels-Ansdell D, et al. Effects of higher- versus lower-protein diets on health outcomes: a systematic review and meta-analysis. *Eur J Clin Nutr*. (2012) 66:780–8. doi: 10.1038/ejcn.2012.37

98. Due A, Toubro S, Skov A, Astrup A. Effect of normal-fat diets, either medium or high in protein, on body weight in overweight subjects: a randomised 1-year trial. *Int J Obes Relat Metab Disord*. (2004) 28:1283–90. doi: 10.1038/sj.ijo.0802767

99. McAuley K, Smith K, Taylor R, McLay R, Williams S, Mann J. Long-term effects of popular dietary approaches on weight loss and features of insulin resistance. *Int J Obes (Lond)*. (2006) 30:342–9. doi: 10.1038/sj.ijo.0803075

100. Clifton PM, Condo D, Keogh JB. Long term weight maintenance after advice to consume low carbohydrate, higher protein diets – a systematic review and meta analysis. *Nutr Metab Cardiovasc Dis*. (2014) 24:224–35. doi: 10.1016/j.numecd.2013.11.006

101. Clifton PM, Keogh JB, Noakes M. Long-term effects of a high-protein weight-loss diet. *Am J Clin Nutr*. (2008) 87:23–9. doi: 10.1093/ajcn/87.1.23

102. Layman DK, Evans EM, Erickson D, Seyler J, Weber J, Bagshaw D, et al. A moderate-protein diet produces sustained weight loss and long-term changes in body composition and blood lipids in obese adults. *J Nutr*. (2009) 139:514–21. doi: 10.3945/jn.108.099440

103. Magkos F. Protein-rich diets for weight loss maintenance. *Curr Obes Rep*. (2020) 9:213–8. doi: 10.1007/s13679-020-00391-0

104. Dansinger ML, Gleason JA, Griffith JL, Selker HP, Schaefer EJ. Comparison of the Atkins, Ornish, Weight Watchers, and Zone diets for weight loss and heart disease risk reduction: a randomized trial. *JAMA*. (2005) 293:43–53. doi: 10.1001/jama.293.1.43

105. Sacks FM, Bray GA, Carey VJ, Smith SR, Ryan DH, Anton SD, et al. Comparison of weight-loss diets with different compositions of fat, protein, and carbohydrates. *N Engl J Med*. (2009) 360:859–73. doi: 10.1056/NEJMoa0804748

106. Ran A, Ellen W, Ad AMM, Hauke S, Erwin GZ, Daisy J. Age-dependent changes in GI physiology and microbiota: time to reconsider? *Gut*. (2018) 67:2213–22. doi: 10.1136/gutjnl-2017-315542

107. Walther B, Lett AM, Bordoni A, Tomás-Cobos L, Nieto JA, Dupont D, et al. GutSelf: Interindividual variability in the processing of dietary compounds by the human gastrointestinal tract. *Mol Nutr Food Res*. (2019) 63:1900677. doi: 10.1002/mnfr.201900677

108. Wilding J. The importance of weight management in type 2 diabetes mellitus. *Int J Clin Pract*. (2014) 68:682–91. doi: 10.1111/ijcp.12384

109. Abete I, Astrup A, Martínez JA, Thorsdottir I, Zulet MA. Obesity and the metabolic syndrome: role of different dietary macronutrient distribution patterns and specific nutritional components on weight loss and maintenance. *Nutr Rev*. (2010) 68:214–31. doi: 10.1111/nure.2010.68.issue-4

110. Jing T, Zhang S, Bai M, Chen Z, Gao S, Li S, et al. Effect of dietary approaches on glycemic control in patients with type 2 diabetes: a systematic review with network meta-analysis of randomized trials. *Nutrients*. (2023) 15:3156. doi: 10.3390/nu15143156

111. Samkani A, Skytte MJ, Kandel D, Kjaer S, Astrup A, Deacon CF, et al. A carbohydrate-reduced high-protein diet acutely decreases postprandial and diurnal glucose excursions in type 2 diabetes patients. *Br J Nutr*. (2018) 119:910–17. doi: 10.1017/S0007114518000521

112. Jakubowicz D, Wainstein J, Landau Z, Ahren B, Barnea M, Bar-Dayan Y, et al. High-energy breakfast based on whey protein reduces body weight, postprandial glycemia and $HbA_{1C\ in}$ type 2 diabetes. *J Nutr Biochem*. (2017) 49:1–7. doi: 10.1016/j.jnutbio.2017.07.005

113. Skytte MJ, Samkani A, Astrup A, Frystyk J, Rehfeld JF, Holst JJ, et al. Effects of carbohydrate restriction on postprandial glucose metabolism, β-cell function, gut hormone secretion, and satiety in patients with type 2 diabetes. *Am J Physiol Endocrinol Metab*. (2021) 320:7–18. doi: 10.1152/ajpendo.00165.2020

114. Krebs JD, Elley CR, Parry-Strong A, Lunt H, Drury PL, Bell DA, et al. The Diabetes Excess Weight Loss (DEWL) Trial: a randomised controlled trial of high-protein versus high-carbohydrate diets over 2 years in type 2 diabetes. *Diabetologia*. (2012) 55:905–14. doi: 10.1007/s00125-012-2461-0

115. Parker B, Noakes M, Luscombe N, Clifton P. Effect of a high-protein, high–monounsaturated fat weight loss diet on glycemic control and lipid levels in type 2 diabetes. *Diabetes Care*. (2002) 25:425–30. doi: 10.2337/diacare.25.3.425

116. Yu Z, Nan F, Wang LY, Jiang H, Chen W, Jiang Y. Effects of high-protein diet on glycemic control, insulin resistance and blood pressure in type 2 diabetes: A systematic review and meta-analysis of randomized controlled trials. *Clin Nutr*. (2020) 39:1724–34. doi: 10.1016/j.clnu.2019.08.008

117. Bramante CT, Lee CJ, Gudzune KA. Treatment of obesity in patients with diabetes. *Diabetes Spectr*. (2017) 30:237–43. doi: 10.2337/ds17-0030

118. Monnier L, Lapinski H, Colette C. Contributions of fasting and postprandial plasma glucose increments to the overall diurnal hyperglycemia of type 2 diabetic patients: variations with increasing levels of HbA(1c). *Diabetes Care*. (2003) 26:881–5. doi: 10.2337/diacare.26.3.881

119. Tay J, Luscombe-Marsh ND, Thompson CH, Noakes M, Buckley JD, Wittert GA, et al. Comparison of low-and high-carbohydrate diets for type 2 diabetes management: a randomized trial. *Am J Clin Nutr*. (2015) 102:780–90. doi: 10.3945/ajcn.115.112581

120. Thomsen MN, Skytte MJ, Astrup A, Deacon CF, Holst JJ, Madsbad S, et al. The clinical effects of a carbohydrate-reduced high-protein diet on glycaemic variability in metformin-treated patients with type 2 diabetes mellitus: a randomised controlled study. *Clin Nutr ESPEN*. (2020) 39:46–52. doi: 10.1016/j.clnesp.2020.07.002

121. Tettamanzi F, Bagnardi V, Louca P, Nogal A, Monti GS, Mambrini SP, et al. A high protein diet is more effective in improving insulin resistance and glycemic variability compared to a mediterranean diet - a cross-over controlled inpatient dietary study. *Nutrients*. (2021) 13:4380. doi: 10.3390/nu13124380

122. Fabricatore AN, Ebbeling CB, Wadden TA, Ludwig DS. Continuous glucose monitoring to assess the ecologic validity of dietary glycemic index and glycemic load. *Am J Clin Nutr*. (2011) 94:1519–24. doi: 10.3945/ajcn.111.020354

123. Baer DJ, Stote KS, Paul DR, Harris GK, Rumpler WV, Clevidence BA. Whey protein but not soy protein supplementation alters body weight and composition in free-living overweight and obese adults. *J Nutr*. (2011) 141:1489–94. doi: 10.3945/jn.111.139840

124. Takahira M, Noda K, Fukushima M, Zhang B, Mitsutake R, Uehara Y, et al. Randomized, double-blind, controlled, comparative trial of formula food containing soy protein vs. milk protein in visceral fat obesity - FLAVO study. *Circ J*. (2011) 75:2235–43. doi: 10.1253/circj.cj-10-1013

125. Anderson JW, Hoie LH. Weight loss and lipid changes with low-energy diets: comparator study of milk-based versus soy-based liquid meal replacement interventions. *J Am Coll Nutr*. (2005) 24:210–6. doi: 10.1080/07315724.2005.10719467

126. Faghih S, Abadi AR, Hedayati M, Kimiagar SM. Comparison of the effects of cows' milk, fortified soy milk, and calcium supplement on weight and fat loss in premenopausal overweight and obese women. *Nutr Metab Cardiovasc Dis*. (2011) 21:499–503. doi: 10.1016/j.numecd.2009.11.013

127. Anderson JW, Fuller J, Patterson K, Blair R, Tabor A. Soy compared to casein meal replacement shakes with energy-restricted diets for obese women: randomized controlled trial. *Metabolism*. (2007) 56:280–8. doi: 10.1016/j.metabol.2006.10.013

128. Mahon AK, Flynn MG, Stewart LK, McFarlin BK, Iglay HB, Mattes RD, et al. Protein intake during energy restriction: effects on body composition and markers of metabolic and cardiovascular health in postmenopausal women. *J Am Coll Nutr*. (2007) 26:182–9. doi: 10.1080/07315724.2007.10719600

129. Melanson K, Gootman J, Myrdal A, Kline G, Rippe JM. Weight loss and total lipid profile changes in overweight women consuming beef or chicken as the primary protein source. *Nutrition*. (2003) 19:409–14. doi: 10.1016/s0899-9007(02)01080-8

130. Neacsu M, Fyfe C, Horgan G, Johnstone AM. Appetite control and biomarkers of satiety with vegetarian (soy) and meat-based high-protein diets for weight loss in obese men: a randomized crossover trial. *Am J Clin Nutr*. (2014) 100:548–58. doi: 10.3945/ajcn.113.077503

131. Abete I, Parra D, Martinez JA. Legume-, fish-, or high-protein-based hypocaloric diets: effects on weight loss and mitochondrial oxidation in obese men. *J Med Food*. (2009) 12:100–8. doi: 10.1089/jmf.2007.0700

132. Aldrich ND, Reicks MM, Sibley SD, Redmon JB, Thomas W, Raatz SK. Varying protein source and quantity do not significantly improve weight loss, fat loss, or satiety in reduced energy diets among midlife adults. *Nutr Res*. (2011) 31:104–12. doi: 10.1016/j.nutres.2011.01.004

133. Li J, Armstrong CLH, Campbell WW. Effects of dietary protein source and quantity during weight loss on appetite, energy expenditure, and cardio-metabolic responses. *Nutrients*. (2016) 8:63. doi: 10.3390/nu8020063

134. Teixeira FJ, Matias CN, Faleiro J, Giro R, Pires J, Figueiredo H, et al. A novel plant-based protein has similar effects compared to whey protein on body composition, strength, power, and aerobic performance in professional and semi-professional futsal players. *Front Nutr*. (2022) 9:934438. doi: 10.3389/fnut.2022.934438

135. Vasei MH, Hosseinpour-Niazi S, Ainy E, Mirmiran P. Effect of dietary approaches to stop hypertension (DASH) diet, high in animal or plant protein on cardiometabolic risk factors in obese metabolic syndrome patients: a randomized clinical trial. *Prim Care Diabetes*. (2022) 16:634–9. doi: 10.1016/j.pcd.2022.09.001

136. Toh DWK, Fu AS, Mehta KA, Lam NYL, Haldar S, Henry CJ. Plant-based meat analogs and their effects on cardiometabolic health: an 8-week randomized controlled trial comparing plant-based meat analogs with their corresponding animal-based foods. *Am J Clin Nutr*. (2024) 119:1405–16. doi: 10.1016/j.ajcnut.2024.04.006

137. Halkjær J, Olsen A, Overvad K, Jakobsen MU, Boeing H, Buijsse B, et al. Intake of total, animal and plant protein and subsequent changes in weight or waist circumference in European men and women: the Diogenes project. *Int J Obes (Lond)*. (2011) 35:1104–13. doi: 10.1038/ijo.2010.254

138. Alhazmi A, Stojanovski E, McEvoy M, Garg ML. Macronutrient intake and type 2 diabetes risk in middle-aged Australian women. Results from the Australian Longitudinal Study on Women's Health. *Public Health Nutr*. (2014) 17:1587–94. doi: 10.1017/S1368980013001870

139. Mirmiran P, Bahadoran Z, Gaeini Z, Azizi F. Habitual intake of dietary L-arginine in relation to risk of type 2 diabetes: a prospective study. *BMC Endocr Disord*. (2021) 21:113. doi: 10.1186/s12902-021-00774-x

140. Smith JD, Hou T, Ludwig DS, Rimm EB, Willett W, Hu FB, et al. Changes in intake of protein foods, carbohydrate amount and quality, and long-term weight change: results from 3 prospective cohorts. *Am J Clin Nutr*. (2015) 101:1216–24. doi: 10.3945/ajcn.114.100867

141. Bujnowski D, Xun P, Daviglus ML, Van Horn L, He K, Stamler J. Longitudinal association between animal and vegetable protein intake and obesity among men in the United States: the Chicago Western Electric Study. *J Am Diet Assoc*. (2011) 111:1150–5. doi: 10.1016/j.jada.2011.05.002

142. Lin Y, Bolca S, Vandevijvere S, De Vriese S, Mouratidou T, De Neve M, et al. Plant and animal protein intake and its association with overweight and obesity among the Belgian population. *Br J Nutr*. (2011) 105:1106–16. doi: 10.1017/S0007114510004642

143. Kim MN, Lo C-H, Corey KE, Luo X, Long L, Zhang X, et al. Red meat consumption, obesity, and the risk of nonalcoholic fatty liver disease among women: Evidence from mediation analysis. *Clin Nutr*. (2022) 41:356–64. doi: 10.1016/j.clnu.2021.12.014

144. Bajracharya R, Katzke V, Mukama T, Kaaks R. Effect of iso-caloric substitution of animal protein for other macro nutrients on risk of overall, cardiovascular and cancer mortality: prospective evaluation in EPIC-Heidelberg cohort and systematic review. *Nutrients*. (2023) 15:794. doi: 10.3390/nu15030794

145. Das A, Cumming R, Naganathan V, Blyth F, Couteur DGL, Handelsman DJ, et al. Associations between dietary intake of total protein and sources of protein (plant vs. animal) and risk of all-cause and cause-specific mortality in older Australian men: The Concord Health and Ageing in Men Project. *J Hum Nutr Diet*. (2022) 35:845–60. doi: 10.1111/jhn.12965

146. Hidayat K, Chen J-S, Wang H-P, Wang T-C, Liu Y-J, Zhang X-Y, et al. Is replacing red meat with other protein sources associated with lower risks of coronary heart disease and all-cause mortality? a meta-analysis of prospective studies. *Nutr Rev*. (2022) 80:1959–73. doi: 10.1093/nutrit/nuac017

147. Sluijs I, Beulens JWJ, van der A DL, Spijkerman AMW, Grobbee DE, van der Schouw YT. Dietary intake of total, animal, and vegetable protein and risk of type 2 diabetes in the European Prospective Investigation into Cancer and Nutrition (EPIC)-NL study. *Diabetes Care*. (2009) 33:43–8. doi: 10.2337/dc09-1321

148. Zhou C, Liu C, Zhang Z, Liu M, Zhang Y, Li H, et al. Variety and quantity of dietary protein intake from different sources and risk of new-onset diabetes: a Nationwide Cohort Study in China. *BMC Med*. (2022) 20:6. doi: 10.1186/s12916-021-02199-8

149. Shang X, Scott D, Hodge AM, English DR, Giles GG, Ebeling PR, et al. Dietary protein intake and risk of type 2 diabetes: results from the Melbourne Collaborative Cohort Study and a meta-analysis of prospective studies. *Am J Clin Nutr*. (2016) 104:1352–65. doi: 10.3945/ajcn.116.140954

150. Song Y, Manson JE, Buring JE, Liu S. A prospective study of red meat consumption and type 2 diabetes in middle-aged and elderly women: The Women's Health Study. *Diabetes Care*. (2004) 27:2108–15. doi: 10.2337/diacare.27.9.2108

151. Sugihiro T, Yoneda M, Ohno H, Oki K, Hattori N. Associations of nutrient intakes with obesity and diabetes mellitus in the longitudinal medical surveys of Japanese Americans. *J Diabetes Investig*. (2019) 10:1229–36. doi: 10.1111/jdi.13010

152. van Nielen M, Feskens EJ, Mensink M, Sluijs I, Molina E, Amiano P, et al. Dietary protein intake and incidence of type 2 diabetes in Europe: the EPIC-InterAct Case-Cohort Study. *Diabetes Care*. (2014) 37:1854–62. doi: 10.2337/dc13-2627

153. Yuan S, Ming-wei L, Qi-qiang H, Larsson SC. Egg, cholesterol and protein intake and incident type 2 diabetes mellitus: Results of repeated measurements from a prospective cohort study. *Clin Nutr*. (2021) 40:4180–6. doi: 10.1016/j.clnu.2021.01.041

154. Chen Z, Franco OH, Lamballais S, Ikram MA, Schoufour JD, Muka T, et al. Associations of specific dietary protein with longitudinal insulin resistance, prediabetes and type 2 diabetes: The Rotterdam Study. *Clin Nutr*. (2020) 39:242–9. doi: 10.1016/j.clnu.2019.01.021

155. Fan M, Li Y, Wang C, Mao Z, Zhou W, Zhang L, et al. Dietary protein consumption and the risk of type 2 diabetes: adose-response meta-analysis of prospective studies. *Nutrients*. (2019) 11:2783. doi: 10.3390/nu11112783

156. Zhao LG, Zhang QL, Liu XL, Wu H, Zheng JL, Xiang YB. Dietary protein intake and risk of type 2 diabetes: a dose-response meta-analysis of prospective studies. *Eur J Nutr*. (2019) 58:1351–67. doi: 10.1007/s00394-018-1737-7

157. Ye J, Yu Q, Mai W, Liang P, Liu X, Wang Y. Dietary protein intake and subsequent risk of type 2 diabetes: a dose-response meta-analysis of prospective cohort studies. *Acta Diabetol*. (2019) 56:851–70. doi: 10.1007/s00592-019-01320-x

158. Schulze MB, Haardt J, Amini AM, Kalotai N, Lehmann A, Schmidt A, et al. Protein intake and type 2 diabetes mellitus: an umbrella review of systematic reviews for the evidence-based guideline for protein intake of the German Nutrition Society. *Eur J Nutr*. (2024) 63:33–50. doi: 10.1007/s00394-023-03234-5

159. Fotouhi Ardakani A, Anjom-Shoae J, Sadeghi O, Marathe CS, Feinle-Bisset C, Horowitz M. Association between total, animal, and plant protein intake and type 2 diabetes risk in adults: a systematic review and dose-response meta-analysis

of prospective cohort studies. *Clin Nutr*. (2024) 43:1941–55. doi: 10.1016/j.clnu.2024.07.001

160. Lamberg-Allardt C, Bärebring L, Arnesen EK, Nwaru BI, Thorisdottir B, Ramel A, et al. Animal versus plant-based protein and risk of cardiovascular disease and type 2 diabetes: a systematic review of randomized controlled trials and prospective cohort studies. *Food Nutr Res*. (2023) 67 (In press). doi: 10.29219/fnr.v67.9003

161. Jackman MR, Steig A, Higgins JA, Johnson GC, Fleming-Elder BK, Bessesen DH, et al. Weight regain after sustained weight reduction is accompanied by suppressed oxidation of dietary fat and adipocyte hyperplasia. *Am J Physiol Regul Integr Comp Physiol*. (2008) 294:1117–29. doi: 10.1152/ajpregu.00808.2007

162. Van Baak MA, Mariman EC. Mechanisms of weight regain after weight loss - the role of adipose tissue. *Nat Rev Endocrinol*. (2019) 15:274–87. doi: 10.1038/s41574-018-0148-4

163. Tremblay F, Marette A. Amino acid and insulin signaling *via* the mTOR/p70 S6 kinase pathway. A negative feedback mechanism leading to insulin resistance in skeletal muscle cells. *J Biol Chem*. (2001) 276:38052–60. doi: 10.1074/jbc.M106703200

164. Khamzina L, Veilleux A, Bergeron S, Marette A. Increased activation of the mammalian target of rapamycin pathway in liver and skeletal muscle of obese rats: possible involvement in obesity-linked insulin resistance. *Endocrinology*. (2005) 146:1473–81. doi: 10.1210/en.2004-0921

165. Krebs M, Krssak M, Bernroider E, Anderwald C, Brehm A, Meyerspeer M, et al. Mechanism of amino acid-induced skeletal muscle insulin resistance in humans. *Diabetes*. (2002) 51:599–605. doi: 10.2337/diabetes.51.3.599

166. Linn T, Santosa B, Grönemeyer D, Aygen S, Scholz N, Busch M, et al. Effect of long-term dietary protein intake on glucose metabolism in humans. *Diabetologia*. (2000) 43:1257–65. doi: 10.1007/s001250051521

167. Weickert MO, Roden M, Isken F, Hoffmann D, Nowotny P, Osterhoff M, et al. Effects of supplemented isoenergetic diets differing in cereal fiber and protein content on insulin sensitivity in overweight humans. *Am J Clin Nutr*. (2011) 94:459–71. doi: 10.3945/ajcn.110.004374

168. Barnard ND, Katcher HI, Jenkins DJ, Cohen J, Turner-McGrievy G. Vegetarian and vegan diets in type 2 diabetes management. *Nutr Rev*. (2009) 67:255–63. doi: 10.1111/nure.2009.67.issue-5

169. Pan A, Sun Q, Bernstein AM, Schulze MB, Manson JE, Willett WC, et al. Red meat consumption and risk of type 2 diabetes: 3 cohorts of US adults and an updated meta-analysis. *Am J Clin Nutr*. (2011) 94:1088–96. doi: 10.3945/ajcn.111.018978

170. Arjmandi BH, Khalil DA, Smith BJ, Lucas EA, Juma S, Payton ME, et al. Soy protein has a greater effect on bone in postmenopausal women not on hormone replacement therapy, as evidenced by reducing bone resorption and urinary calcium excretion. *J Clin Endocrinol Metab*. (2003) 88:1048–54. doi: 10.1210/jc.2002-020849

171. Kerstetter JE, O'Brien KO, Caseria DM, Wall DE, Insogna KL. The impact of dietary protein on calcium absorption and kinetic measures of bone turnover in women. *J Clin Endocrinol Metab*. (2005) 90:26–31. doi: 10.1210/jc.2004-0179

172. Kerstetter JE, O'Brien KO, Insogna KL. Dietary protein, calcium metabolism, and skeletal homeostasis revisited. *Am J Clin Nutr*. (2003) 78:584–92. doi: 10.1093/ajcn/78.3.584S

173. Mangano KM, Walsh SJ, Kenny AM, Insogna KL, Kerstetter JE. Dietary acid load is associated with lower bone mineral density in men with low intake of dietary calcium. *J Bone Miner Res*. (2014) 29:500–6. doi: 10.1002/jbmr.2053

174. Friedman AN. High-protein diets: potential effects on the kidney in renal health and disease. *Am J Kidney Dis*. (2004) 44:950–62. doi: 10.1053/j.ajkd.2004.08.020

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Emerging roles of lipid and metabolic sensing in the neuroendocrine control of body weight and reproduction

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The hypothalamus lies at the intersection of brain and hormonal mechanisms governing essential bodily functions, including metabolic/body weight homeostasis and reproduction. While metabolism and fertility are precisely regulated by independent neuroendocrine axes, these are tightly connected, as reflection of the bidirectional interplay between the energy status of the organisms and their capacity to reproduce; a connection with important pathophysiological implications in disorders affecting these two crucial systems. Beyond the well-characterized roles of key hormones (e.g., leptin, insulin, ghrelin) and neuropeptides (e.g., melanocortins, kisspeptins) in the integral control of metabolism and reproduction, mounting evidence has pointed out a relevant function of cell energy sensors and lipid sensing mechanisms in the hypothalamic control of metabolism, with prominent roles also for metabolic sensors, such as mTOR, AMPK and SIRT1, in the nutritional regulation of key aspects of reproduction, such as pubertal maturation. We provide herein a synoptic overview of these novel regulatory pathways, with a particular focus on their putative function in the metabolic control of puberty, and delineate new avenues for further exploration of the intricate mechanisms whereby metabolism and reproduction are tightly connected.

KEYWORDS

lipid sensing, reproduction, mTOR, AMPK, sirtuins, ceramides, fatty acids, Kiss1

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1 Introduction: neuroendocrine mechanisms for the control of body weight and reproduction

Metabolism and reproduction are two closely related bodily functions essential for the survival of the organism and the species, respectively. An unequivocal indication of this interaction is that significant disruptions in energy balance, ranging from malnutrition to obesity, are often associated not only with several metabolic alterations, but also with pubertal disorders and reduced reproductive capacity in adulthood (1, 2). Similarly, certain reproductive disorders, such as hypogonadism, may exacerbate an altered metabolic state, such as that bound to obesity (3).

Although the mechanisms linking energy balance and reproduction are intricate and not fully understood yet, it is known that they primarily have a neuroendocrine basis. This involves the complex interaction between specific peripheral signals, particularly metabolic hormones, and molecules generated by the central nervous system, mainly neurotransmitters and neuropeptides (2, 4). The coordination of this interaction occurs primarily in the hypothalamus, a small brain region located below the thalamus that operates as an integrator hub for these signals and produces appropriate homeostatic responses to support the proper functioning of our organism (5).

Leptin, ghrelin, and insulin are essential hormones regulating energy balance and reproductive function. While these hormones have effects at local and peripheral levels, it has been conclusively documented that their primary influence on energy balance and reproductive function occurs through their actions at the hypothalamic level (2, 4, 6). These hormones are now known to directly or indirectly affect specific groups of neurons that play a key role in controlling metabolism and reproduction, which are mainly located in the hypothalamic arcuate nucleus (ARC) or preoptic areas (2). These include: (i) orexigenic neurons co-expressing neuropeptide Y (NPY) and agouti-related peptide (AgRP) – i.e., NPY/AgRP neurons; (ii) anorexigenic neurons co-expressing the peptide products of proopiomelanocortin (POMC), such as alpha-melanocyte-stimulating hormone (α-MSH), and the cocaine- and amphetamine-regulated transcript (CART) – i.e., POMC/CART neurons; (iii) neurons producing gonadotropin-releasing hormone (GnRH), which are essential for the ultimate activation of reproductive axis at puberty and its regulation in adulthood; and (iv) neurons modulating the activity of GnRH neurons, including prominently neurons producing the neuropeptides kisspeptins – named Kiss1 neurons, which are potent stimulators of the reproductive axis (7).

In addition, neuronal populations at other key hypothalamic nuclei, such as the ventromedial (VMH) and dorsomedial (DMH) hypothalamic nuclei, as well as the lateral hypothalamus, have been shown to participate in the control of feeding, energy balance and thermoregulation (8), as well as their interplay with reproductive hormones, such as estrogens (9). Similarly, the relevance of the suprachiasmatic nucleus (SCN) in the regulation of circadian rhythms modulating reproductive and metabolic function (10), as well as the key role of the ventral premammillary nucleus (PMV), as an important integrating node of metabolic and reproductive status, have been documented (6). Altogether, these heterogeneous neuronal components and the peripheral signals they interact with constitute the main neuroendocrine pathways involved in controlling energy balance and reproduction.

In the same vein, a very recent study, which integrated information from seventeen single-cell RNA sequencing datasets into a mouse hypothalamic cell atlas (named HypoMap), has highlighted the complex heterogeneity of the neuronal populations and subpopulations within the hypothalamus and the existence of differential transcriptional responses to specific conditions of metabolic stress, such as fasting (11). This heterogeneity is not restricted to neuronal populations, but affects also glial cells, including astrocytes, oligodendrocytes and their precursor cells, microglia, ependymal cells and tanycytes, which are highly relevant in the modulation of hypothalamic circuits (12). Indeed, compelling evidence has been presented for the involvement of these glial cells in the control of energy balance, including modulation of leptin responses, nutrient sensing and metabolite transport (12, 13), and more recently in the modulation of the reproductive axis by metabolic cues, via kisspeptin-mediated astrocyte signaling (14). Altogether, these heterogeneous neuronal and glial components illustrate the high degree of complexity and sophistication of the mechanisms involved in controlling energy balance and reproduction.

Notably, while the biological actions of the metabolic hormones and neuropeptides indicated above have been extensively characterized in the last three decades, in recent years, novel molecular mechanisms for the homeostatic control of bodily metabolism and reproduction, operating at the hypothalamus, particularly in some of the above neuronal systems, have been unveiled. Part of these recent developments are summarized here, with a specific focus on new pathways putatively involved in the metabolic control of puberty and fertility.

2 Role of energy sensors in the hypothalamic control of energy homeostasis

Energy sensors are molecular and cellular systems that perceive the energetic state of a cell or, when operating in specific cellular pathways, of an organism. A caloric nutrient sensor is typically a protein that identifies a specific macronutrient, prompting a cellular response that alters nutrient distribution, animal feeding behavior and/or its metabolic rate. These sensors can operate intracellularly, detecting nutrient flux within metabolic pathways, or extracellularly, perceiving nutrients in the surrounding environment (15, 16). The intricate network of hormonal and neural pathways linking multiple nutrient-sensing organs —such as the intestine, pancreas, liver, adipose tissue, and the brain— suggests a vast communication among nutrient-sensing cells across diverse organ systems (17). Among others, recent studies have demonstrated that pathways, such as AMPK, mTOR and SIRT1, the latter as prominent member of the sirtuin family, are pivotal elements in adaptive responses to changes in body nutritional status and energy reserves, therefore contributing to modulate the energetic and reproductive status of the organism (18).

2.1 AMP-activated protein kinase and the neuroendocrine control of metabolism

AMPK is an ubiquitous, highly-conserved kinase, composed of one catalytic subunit (α) and two regulatory subunits (β and γ) (19). AMPK acts as a *bona fide* cell energy sensor, since its activity is driven by changes in the abundance of adenine nucleotides, i.e., AMP, but also ADP and ATP, within the cells. Thus, conditions of energy deprivation result in enhanced activity of AMPK, which in turn represses ATP-consuming phenomena, while it enhances ATP-producing processes. This leads to the restoration of AMP: ATP and ADP: ATP ratios, therefore contributing to energy homeostasis at the cellular level. Importantly, besides this function, acting in specific hypothalamic circuits, AMPK plays a fundamental role in the maintenance of whole-body energy balance, by promoting feeding and catabolic reactions, whereas it suppresses anabolic pathways systemically (17, 20).

In line with this systemic function, in recent years, AMPK-mediated pathways in the hypothalamus have been shown to operate as the main canonical route whereby multiple endocrine factors actively participate in the precise regulation of whole-body metabolism and weight homeostasis. This pivotal role as metabolic mediator has been documented not only for essential metabolic hormones, such as those coming from the adipose tissue (leptin), gut (ghrelin, glucagon-like peptide-1, GLP-1), gonads (estrogens) and thyroid gland (thyroid hormones), but also metabolites, such as glucose (21). Thus, AMPK activity in the hypothalamus seemingly orchestrates a diversity of responses, including stimulation of food intake and modulation of food preferences, regulation of glucose homeostasis (22), suppression of brown adipose tissue thermogenesis (23), and regulation of hepatic function (23), just to highlight its most prominent functions. Interestingly, according to experimental studies, these activities appear to display a considerable degree of nucleus-specificity within the hypothalamus, so that while the function of AMPK to enhance food intake seems to reside mainly in the ARC, the capacity of AMPK to suppress thermogenesis is located mainly at the VMH (17). While the cellular substrate for these differential actions is yet to be fully characterized, it has been shown that AMPK signaling within ARC POMC and AgRP neurons is essential for feeding control (24) whereas neurons expressing steroidogenic factor 1 (SF1) in the VMH play a crucial role in mediating the thermogenic-regulatory actions of AMPK, via sympathetic regulation of brown adipose tissue activity (25).

2.2 The mammalian target of rapamycin and the neuroendocrine control of metabolism

mTOR is an evolutionary conserved factor belonging to the PI3K-related kinase family. It forms two multimeric complexes, mTORC1 and mTORC2, through their respective interactions with integral proteins, RAPTOR and RICTOR (26). Activation of mTORC1 leads to the inactivation of mTORC2 and vice versa; thus, the mTORC1/mTORC2 tandem represents an essential component of a negative feedback loop to ensure proper signaling. At the cellular level, mTORC1 senses and becomes activated by nutrient abundance, by detecting different cues, from amino acids to cell stress and energy status, therefore linking these to cell growth and proliferation (27). Accordingly, mTOR and AMPK operate in a reciprocal manner, so that conditions that activate mTOR suppress AMPK activity, and vice versa (28).

At the systemic level, mTOR signaling in the mediobasal hypothalamus becomes activated by leptin, as a signal of energy abundance (29), and mediates, at least partially, the feeding-promoting effects of ghrelin, as putative signal of energy deficit (30), thereby coordinating appropriate feeding and metabolic responses (31). In addition, disruption of mTORC2 in neurons led to increased fat composition, adiposity, and impaired glucose tolerance, while deletion of RICTOR in POMC neurons resulted in obesity, hyperphagia, and glucose intolerance (32), therefore supporting a relevant role of central mTOR signaling in the integral control of metabolism. Of note, as it is the case also for AMPK, the hypothalamic actions of mTOR seem to display some degree of nucleus- and cell-specificity, as demonstrated by the fact that mTOR activity in the ARC and VMH was oppositely regulated by fasting and leptin deficiency (33). These findings, together with the interplay of brain mTOR with other metabolic hormones, such as nesfatin-1 (34), illustrate the complexity of the mode of action of mTOR-dependent pathways in the central control of metabolism.

2.3 SIRT1 and the neuroendocrine control of metabolism

Sirtuins are a family of regulatory proteins, highly conserved across evolution, whose initial member is Sir2, identified in *Saccharomyces cerevisiae* (35). In mammals, up to seven sirtuins, including nuclear and mitochondrial proteins, have been identified; SIRT1 being the most prominent and best characterized member of the sirtuin superfamiliy (36). From a functional standpoint, SIRT1 acts as a NAD^+-dependent deacetylase (37), with capacity to erase acetylation marks from histones and other protein targets. Thereby, SIRT1 can epigenetically modulate multiple biological processes, seemingly including lifespan and healthy ageing (35). Notably, SIRT1, as well as other sirtuins, operate as a genuine cell energy sensor, since their activation is dictated by fluctuations in the cellular levels of the cofactor, NAD^+, and its related intermediary products, NADH and nicotinamide. Thus, conditions of energy deprivation, such as caloric restriction, that enhance NAD^+/NADH and NAD^+/nicotinamide ratios, are known to cause accumulation and activation of SIRT1 in different tissues, including the brain.

Indeed, while SIRT1 is expressed in multiple peripheral tissues, where it is involved in different metabolic adaptive responses in the pancreas, muscle, liver and adipose tissue, SIRT1 is known to be expressed also in the CNS, where it has been shown to participate in the regulation of systemic homeostatic responses, including food intake and energy expenditure (38). In fact, in adulthood, high expression levels of SIRT1 have been reported in different hypothalamic nuclei, including the VMH, dorsomedial and

paraventricular nuclei, as well as the ARC (39). On the latter, SIRT1 has been shown to be expressed and operate in essential neuronal populations in the control of metabolic homeostasis, such as POMC and AgRP neurons (38, 40). Moreover, hypothalamic SIRT1 signaling has been shown to participate in conveying the effects of key metabolic hormones, such as ghrelin (41), while SIRT1 in POMC neurons mediates, at least partially, the effects of leptin on PI3K signaling in this neuronal population and leptin-induced remodeling of white adipose tissue (42). Of note, SIRT1 protein levels are elevated in the hypothalamus in conditions of energy deprivation (39); a profile that is mirrored by hypothalamic AMPK levels, which are also elevated under energy deficit. In fact, SIRT1 and AMPK are both fuel-sensing molecules that reciprocally activate each other, therefore contributing to mediate metabolic adaptations to conditions such as energy deprivation (43). Importantly, the hypothalamic actions of SIRT1 are not restricted to ARC neuronal populations; thus, ablation of SIRT1 in SF1 neurons, abundantly expressed in the VMH, is bound to metabolic perturbations, while over-expression of SIRT1 in this neuronal population protects from diet-induced obesity and insulin resistance (44). In addition, other sirtuins, such as SIRT3 and SIRT6, have been shown to operate at the hypothalamic, and particularly in POMC neurons, to contribute to energy homeostasis and adaptive responses to lessen the metabolic impact of obesity (38, 45, 46).

3 Role of energy sensors in the hypothalamic control of reproduction

Hypothalamic circuits engaging cellular energy sensors, such as AMPK, mTOR and SIRT1, have been shown also to participate in the metabolic regulation of the neuronal pathways governing puberty onset and the reproductive axis. Indeed, while GnRH neurons in the basal forebrain operate as the main output pathway for the brain control of puberty and fertility, the secretory activity of GnRH neurons is exquisitely dependent on the regulatory actions of upstream regulatory circuits, including prominently Kiss1 neurons, producing kisspeptins, which participate in conveying the modulatory effects of metabolic cues on GnRH neurons. Accordingly, the activity of these sensors has been shown to regulate, either directly or indirectly these key neuronal populations, as a means to funnel the influence of nutritional and metabolic signals to the centers governing the reproductive axis.

3.1 AMPK and the metabolic control of puberty and reproduction

The potential function of AMPK in the regulation of the reproductive axis by metabolic cues has been explored in recent years, with a particular focus on its role in the modulation of Kiss1 and GnRH neurons. In line with the capacity of conditions energy deficit to enhance AMPK activity in the hypothalamus, pharmacological activation of brain AMPK has been shown to impair ovarian cyclicity (47) and substantially delayed puberty onset in female rats (48). Initial fragmentary evidence suggested that this effect might involve an inhibition of the Kiss1 system since the adipose hormone, adiponectin, was shown to suppress *Kiss1* expression via induction of AMPK activity in the GnRH cell line, GT1-7 (49). This possibility was later documented by a body of experimental evidence from genetically-modified mouse lines. Thus, congenital ablation of the AMPKα2 subunit from Kiss1-expressing cells resulted in resilience to the inhibitory impact of fasting (50), supporting the view that AMPK activity in Kiss1 neurons conveys a negative influence in conditions of food deprivation bound to the inhibition of the reproductive axis. In the same line, we have demonstrated that AMPK signaling in Kiss1 neurons has a discernible role in the metabolic control of puberty, since conditional elimination of the AMPKα1 subunit from Kiss1 neurons protected pubertal female mice from the delay in the onset of puberty induced by post-weaning subnutrition (48). In good agreement, our data in immature female rats documented that pharmacological activation of central AMPK suppressed *Kiss1* expression in the ARC, together with its action in terms of induction of delayed puberty (48).

Interestingly, the activity of AMPK in the metabolic modulation of puberty and the reproductive axis is not restricted to its action in Kiss1 neurons, but also involves regulatory effects in GnRH neurons. Thus, conditional ablation of AMPKα1 from GnRH neurons resulted in accelerated puberty and enhanced responses to exogenous kisspeptin, both in pubertal and adult mice, suggesting that, in normal conditions, AMPK is also suppressing GnRH activity (51). In addition, mice lacking AMPK activity in GnRH neurons also displayed partial resistance to the suppressive impact of energy deprivation on the gonadotropic axis (51). Of note, the nutritional control of GnRH neurons also involves the regulatory actions of the G protein-coupled-receptor kinase-2, GRK2, as putative negative modulator of kisspeptin receptor in situations of subnutrition (52). Thus, conditions of energy deficit can suppress GnRH neurons via AMPK- and GRK2-dependent mechanisms.

3.2 mTOR and the metabolic control of puberty and reproduction

As a putative signal for energy abundance, positively modulated by leptin, the eventual role of mTOR signaling in the hypothalamus in the metabolic control of puberty was explored by our group over a decade ago (53). Indeed, our experimental data in female rats documented that intact brain signaling via mTOR is mandatory for normal pubertal progression. Moreover, pharmacological blockade of central mTOR by rapamycin not only suppressed ARC expression of *Kiss1*, which is a major puberty-promoting signal, but prevented also the permissive effects of leptin on puberty onset. Thus, in immature female rats subjected to chronic subnutrition, the pubertal delay induced by the state of negative energy balance could be rescued by simultaneous treatment with leptin, but this permissive response was abrogated by co-administration of

rapamycin, as a means to inhibit mTOR activity (54). Admittedly, however, it remains unsolved if mTOR conducts such permissive/positive effect directly in Kiss1 neurons and/or operates in upstream afferents to Kiss1 cells. Notwithstanding, this evidence points to a major role of brain mTOR signaling in the metabolic control of puberty.

In this context, the function of several up-stream regulators of the mTOR pathway in the control of Kiss1 neurons has been more recently explored using functional genomics. Conditional ablation of the catalytic subunit of PI3K in Kiss1 neurons has been shown to reduce ARC kisspeptin content and to suppress reproductive function in mice, predominantly in females (55). Importantly, PI3K has been proposed as an integratory hub for conveying the actions of metabolic hormones with key roles in reproductive function, such as leptin. More recently, the function of PTEN (for *phosphatase and tensin homolog*) has been studied in Kiss1 neurons using also genetic ablation approaches; PTEN is known to inhibit PI3K activity. Thus, conditional elimination of PTEN from Kiss1 neurons caused hypertrophy of Kiss1 neurons and enhanced kisspeptin fiber density in mice, which was associated with exaggerated mTOR activity, especially in females (56). From a functional standpoint, these responses were linked to a situation of refractoriness to the negative impact of fasting on luteinizing hormone (LH), as main hormonal signal for gonadal stimulation. These findings are compatible with a function of PTEN to inhibit the PI3K/mTOR pathway in Kiss1 neurons, whereby it can contribute to the suppression of the reproductive axis in situations of nutritional deprivation.

3.3 SIRT1 and the metabolic control of puberty and reproduction

In line with its putative role as co-regulator of AMPK and fuel-energy sensor at the hypothalamus, SIRT1 has been documented to participate in the control of central components of the reproductive axis. The first evidence for such a reproductive dimension came from observations in Sirt1 null mice, which documented a state of hypogonadotropic hypogonadism, due to impaired migration of GnRH neurons (57), that renders Sirt1 KO mice infertile (58), due to a dramatic reduction in the number of GnRH neurons (57) and in the levels of circulating gonadotropins (59). This function of SIRT1, however, seems to be engaged in early developmental events, rather than dynamic regulatory actions linked to fluctuations in the whole-body metabolic status. The latter, however, has been documented by the demonstration of a relevant role of hypothalamic SIRT1, particularly in ARC Kiss1 neurons, in the regulation of puberty and its modulation by nutritional cues.

Expression analyses in immature female rats documented that hypothalamic SIRT1 protein levels decrease, whereas *Kiss1* expression increases during the transition between the infantile and pubertal period. Hypothalamic SIRT1 levels increased in models of pubertal undernutrition, bound to pubertal delay, whereas conditions of early obesity, linked to accelerated puberty, were associated with decreased SIRT1 content in the hypothalamus (60). Interestingly, SIRT1 protein content in Kiss1 neurons mirrored these profiles in the models of subnutrition (with increased SIRT1 levels) and obesity (with decreased SIRT1 content). In good agreement, enhanced SIRT1 activity, caused either by a pharmacological agent or virogenetic over-expression in the ARC, delayed puberty onset in female rats; a response that was associated with a suppression of *Kiss1* expression after pharmacological activation of SIRT1. Overall, this evidence suggests that SIRT1 actually operates as a repressor of *Kiss1*, which is modulated by the nutritional status, and can transduce part of the modulatory effects of either over- or undernutrition on pubertal timing.

The molecular substrate for the regulatory effects of SIRT1 on puberty is likely connected with its capacity to epigenetically modify the chromatin landscape of the *Kiss1* promoter, mainly in the ARC. Thus, during the normal pubertal transition, SIRT1 is evicted from the *Kiss1* promoter, allowing also the removal of other epigenetic repressors, such as EED, therefore adopting a permissive chromatin configuration that enhances *Kiss1* transcription. In conditions of accelerated puberty due to early over-feeding, this eviction is advanced, leading to precious elevation of *Kiss1* expression. In contrast, in conditions of subnutrition, removal of SIRT1 from the *Kiss1* promoter is deferred and this locks the chromatin landscape in a repressive configuration that reduces *Kiss1* expression and, thereby, delays puberty (60). Therefore, SIRT1 represents a link between nutritional status and the epigenetic machinery regulating the *Kiss1* promoter and pubertal maturation. Of note, while no evidence has been presented for a similar role of hypothalamic SIRT1 in the nutritional control of adult reproductive function, our preliminary observations strongly suggest that SIRT1 signaling in Kiss1 neurons in the rostral hypothalamus in adult female rats, that play a prominent role in the control of ovulation, is also relevant for the suppression of the pre-ovulatory surge of gonadotropins, as major hormonal trigger of ovulation, in conditions of energy deficit. Finally, it is interesting to note that over-expression of a mutant form of SIRT1, devoid of deacetylase activity, in astrocytes lowered *Kiss1* expression and perturbed reproductive function, pointing out that SIRT1 signaling in astroglia might also regulate the Kiss1 system in mice (61). Whether, as is the case in energy homeostasis, other members of the SIRT family participate also in the control of the reproductive axis and its modulation by metabolic cues, is yet to be elucidated.

4 Role of lipid sensing mechanisms in the hypothalamic control of energy homeostasis

Besides the role of hypothalamic energy sensors in the control of metabolic homeostasis and the nutritional modulation of puberty and fertility, emerging evidence supports that relevant hypothalamic metabolic pathways, including those governing feeding and body weight, may sense lipid nutrients and lipid mediators, driving also adaptive responses to maintain energy homeostasis. This phenomenon is termed hypothalamic "lipid sensing", whose molecular mechanisms and interactive players

are yet to be fully disclosed (62). In this context, there is a growing body of evidence supporting that fatty acid (FA) sensing in hypothalamic neurons provides signals regarding the metabolic state of the body, therefore enabling precise adjustments for whole-body energy homeostasis (63). Among the putative mechanisms involved, FA receptors have been recently recognized as relevant players in this phenomenon, since these are capable to bind and/or transduce the actions of FAs (64), likely providing an additional layer of sophistication to the brain systems for fine-tuning metabolism. In addition, other factors involved in lipid sensing, including transporters, nuclear receptors and lipid mediators, also contribute to the hypothalamic control of metabolic homeostasis, as briefly summarized in this section.

4.1 Hypothalamic FA receptors and the neuroendocrine control of metabolism

FAs are indispensable constituents of the plasma membrane and a highly efficient energy source. In addition, free FA (FFA) are of special relevance due to their capacity to operate as signaling molecules, with regulatory actions on gene expression and energy homeostasis at various physiological and pathological conditions (65). The regulatory actions of FFAs are mediated by signaling pathways initiated upon binding to FFA receptors (FFAR) present in the cell membrane (66). Different members of the FFAR family are abundantly expressed in multiple metabolic tissues and display ligand specificity by classes of FFA, therefore operating as genuine lipid sensors, able to trigger adaptive metabolic responses (65).

Among FFARs, receptors for long-chain fatty acids (LCFAs; with a chain length of 14-22 carbon atoms) have been shown as key players in the regulation of energy balance. Solid evidence supports that these receptors contribute to sense fluctuations of LCFA in the hypothalamus, with a prominent role in the regulation of whole-body energy metabolism (62, 67). In this context, FFAR4 (aka, GPR120), a membrane GPCR exclusively activated by LCFAs, is particularly relevant in the control of whole-body energy homeostasis (66). It is expressed in several metabolic tissues, including the brain, where FFAR4 has been detected in the hypothalamus, including the ARC (68). Central activation of FFAR has been shown to acutely decrease food intake (68), while administration of the LCFA, docosahexaenoic acid (DHA), prevented the inflammatory state induced by TNF-alpha in an hypothalamic cell line, rHypoE-7, that abundantly expresses FFAR4 (69). While these data may suggest that this FFAR may mediate the beneficial metabolic effects of omega-3 FAs, chronic administration of a FFAR4 agonist did not change energy expenditure or body weight in mice fed high-fat content diet (68).

Another important LCFA receptor is FFAR1 (aka, GPR40), which can be activated not only by LCFAs but also by medium-chain fatty acids (MCFAs, with a chain length of 6-14 carbons). FFAR1 participates in a wide range of physiological functions and its expression has been reported in several tissues, including the CNS. Of note, FFAR1 has been shown to be widely expressed in hypothalamic neurons directly involved in the control of energy homeostasis (64, 70). In this context, a recent study has documented that central pharmacological activation of FFAR1 in diet-induced obese mice decreased body weight and increased energy expenditure, while virogenetic knockdown of FFAR1 in ARC POMC neurons of obese mice evoked hyperphagia and body weight gain, as well as the development of hepatic insulin resistance and steatosis (70). These data highlight the relevance of hypothalamic FFAR1 signaling in the control of adult metabolic homeostasis.

Another MCFA receptor, GPR84, has been found to be expressed also in several metabolic tissues, including the brain (71), where its presence within the hypothalamus has been reported (64). While the information of the roles of GPR84 in the central control of metabolic homeostasis remains scarce, it has been shown that icv treatment with a GPR84 agonist reduced food intake in the rainbow trout together with an increase in hypothalamic mRNA levels of POMC and CART, and a decrease in NPY and AgRP levels (71). Further studies are needed to address the putative role of this FFAR as a hypothalamic lipid sensor in mammals.

Finally, FFARs sensitive to short-chain fatty acids (SCFAs; up to 6 carbon atoms), produced from the fermentation of dietary fibers by the gut microbiota (72), have been shown also to participate in the regulation of various physiological processes, including the maintenance of energy balance. These include FFAR2 (aka, GPR43) and FFAR3 (aka, GPR41), whose expression has been reported in several tissues, including the brain (65). In this context, FFAR2 and FFAR3 are expressed in the hypothalamus of rodents (73, 74). Although the exact mechanism whereby hypothalamic FFAR2/3 exert their metabolic actions is yet to be fully clarified, it has been shown that high fat diet exposure results in increased expression of FFAR3 in the PVN, associated with decreased butyrate levels, as putative contributing factor for development of inflammation and hypertension, while virogenetic silencing of FFAR3 in the PVN attenuated these adverse responses, i.e., tissue inflammation and hypertension, in rats (75).

4.2 Lipid transporters and nuclear receptors and the neuroendocrine control of metabolism

Besides FFARs, elements involved in the translocation of certain FAs inside the cell or the intracellular sensing of lipid species have been suggested to serve a role as lipid sensors themselves. Among these, CD36 (for *cluster of differentiation 36*) is a transmembrane protein expressed in various cell types, including hypothalamic neurons and astrocytes (76). CD36 is primarily known for its dual capacity as both facilitator of LCFAs transport and initiator of signaling cascades upon FA binding (77). Its role as hypothalamic lipid sensor is supported by its documented function as the main mediator of FFA actions in the VMH, as well as its prominent contribution to lipid sensing in glucosensing neurons in the ARC (76). Moreover, depletion of CD36 in the VMH/ARC region in rats fed on high-fat diet caused subcutaneous fat accumulation and increased leptin levels, together with insulin resistance, without an overall effect on food intake and body weight (78).

In addition, FA transport proteins (FATPs), a family of six transmembrane transporters (FATP1-FATP6) involved in the

cellular uptake of FAs and their acylation (76), also contribute to mediating part of the actions of FFA to modulate metabolism, energy homeostasis, and lipid storage. Expression of FATP1 has been detected in neurons of the VMH (63), and *in vitro* studies strongly suggest that FATP1 substantially contributes to the brain uptake of lipid species, such as DHA and oleic acid (OA) (79, 80). Given the proven role of changes in OA and DHA upon hypothalamic neuronal populations, such as POMC and AgRP neurons, it is tenable to consider FATP1 as key component in central lipid sensing. Furthermore, FATP4 expression has been detected in neurons and astrocytes of the VMH in mice (63), and virogenetic silencing of FATP4 in the VMH increased body weight, food intake, fat mass and leptin levels in mice.

Once inside the cell, FAs can function as signals of energy status by acting on nuclear receptors, also involved in lipid sensing, which upon binding operate as transcriptional factors regulating the expression of genes involved in lipid metabolism and energy homeostasis (81). The most prominent example is the family of peroxisome proliferator-activated receptors (PPARs), ligand-activated nuclear receptors that play a crucial role in regulating essential physiological functions, such as glucose and lipid metabolism, as well as energy balance. Upon FA binding, PPARs are translocated to the nucleus and heterodimerize with another nuclear receptor, the retinoid X receptor (RXR), acting as transcription factors by binding to peroxisome proliferator response elements (PPREs) which allows the heterodimer to activate or repress transcription (64). Three different PPAR isoforms -PPARα, PPARβ/δ and PPARγ- have been identified, and their involvement in lipid metabolism has been well documented. While all PPAR isoforms have been detected in the CNS, their expression levels differ among the different isoforms, suggesting distinct roles in regulating metabolism and energy homeostasis. The role of PPARγ as lipid sensor seems to be especially relevant, as its expression has been reported in hypothalamic areas involved in energy homeostasis and feeding behavior (82), while PPARγ levels in AgRP/NPY neurons are sensitive to the nutritional state (83). Moreover, central overexpression of PPARγ in diet-induced obese mice resulted in decreased ghrelin and NPY mRNA levels, while POMC mRNA levels were increased (84). On the contrary, deletion of PPARγ in POMC neurons in high-fat diet fed mice attenuated hyperphagia, increased energy expenditure, and protected from obesity and leptin resistance (85). All these data attest to a prominent role of PPARγ in hypothalamic lipid sensing and metabolic control.

4.3 Hypothalamic lipid mediators and the neuroendocrine control of metabolism

In a broad sense, another component of the lipid sensing mechanisms involves lipid species, other than FFA, with signaling capacities and ability to modulate central pathways governing metabolism. A prominent example is hypothalamic ceramides, lipid molecules composed of a sphingosine moiety bound to FA with a range of chain length, from 14 to 30 carbon atoms (86). Besides the proven role of ceramide accumulation in peripheral tissues in the pathophysiological control of metabolism, compelling evidence, gathered in the last decade, has documented a relevant role for hypothalamic ceramides, in connection with ER stress responses, in mediating the impact of adverse metabolic conditions, such as obesity, at the whole-body level. In fact, ceramide accumulation in the VMH, coupled to ER stress, has been shown to decrease thermogenesis and promote body weight gain and insulin resistance (87). Furthermore, hypothalamic ceramide signaling participates in mediating the metabolic actions of key hormonal regulators, including leptin, ghrelin and estrogens; for instance, the capacity of leptin and estrogens to decrease the hypothalamic levels of ceramides has proven relevant for mediating their effects in terms of induction of thermogenesis and reduction of body weight (87, 88). On the other hand, the pathogenic role of central ceramide accumulation in promoting metabolic perturbations is documented by the fact that inhibition of ceramide synthesis in the brain can ameliorate insulin resistance in leptin-receptor mutant Zucker rats (89), while blockade of ceramide synthesis in the VMH can improve the metabolic profile in rat models of obesity (87). Similarly, CerS6-mediated ceramide synthesis in hypothalamic neurons, as those expressing POMC or SF1, likely contributes to the metabolic deregulation induced by obesogenic diets (90), while ceramides participate also in other relevant brain functions, such as myelination (91).

Finally, bile acids (BA), as derivatives of the lipid precursor, cholesterol, synthesized in the liver (primary BA) and later transformed into secondary BA by the gut microbiota, have gained momentum as putative metabolic mediators, acting in part via hypothalamic circuits (92). Initially considered merely as detergent molecules essential for intestinal lipid absorption, BAs are known to operate via specific nuclear and cell-surface receptors to conduct a variety of regulatory effects (93). Indeed, BAs have been detected in the brain at a concentration that correlates with their circulating levels (94), and BAs have been shown to activate their receptors also in the hypothalamus (95), therefore supporting a role of BA signaling in the mechanisms for central lipid sensing.

Indeed, recent studies have demonstrated that the BA-selective, G protein-coupled receptor, TGR5, operates at the hypothalamus to convey part of the metabolic actions of BAs. Thus, hypothalamic BA content is decreased in obese mice and virogenetic-mediated suppression of TGR5 in the mediobasal hypothalamus promoted obesity development. Conversely, central activation of TGR5 reduced body weight (95). Moreover, specific deletion of TGR5 in AgRP neurons of mice has been recently shown to increase in food intake (96), pointing to the contribution of this hypothalamic pathway for the anorectic effects of BAs.

5 Role of lipid sensing mechanisms in the hypothalamic control of reproduction

While our knowledge of the role of hypothalamic lipid sensing in the control of whole-body homeostasis has substantially expanded during the last decade, whether analogous mechanisms operate in the metabolic regulation of puberty and fertility remains

largely unexplored. From a conceptual standpoint, such intersection between central lipid sensing and the centers governing the reproductive axis is tenable, given the sensitivity of the pathways controlling puberty and fertility to nutritional and metabolic cues, and the proven interplay between reproductive- and metabolic-regulatory circuits within the hypothalamus. Indeed, emerging evidence suggests a role of specific lipid mediators in the perturbations of puberty in conditions of early-obesity (97), while hypothalamic PPARγ seems to modulate part of reproductive responses to high-fat diet in mice (82). Yet, the putative "reproductive" roles of other components of the hypothalamic lipid sensing mechanisms have not been studied yet.

Our group has provided evidence for a novel brain pathway, involving *de novo* ceramide synthesis at the PVN, in the metabolic control of puberty and its alterations after early-onset obesity in rats. Thus, as it is the case in adulthood, the hypothalamic ceramide content was increased in pubertal female rats subjected to postnatal overfeeding, displaying early obesity, which was associated with accelerated puberty. Activation of central *de novo* ceramide synthesis in immature female, but not male rats, also resulted in advancement of the age of puberty onset despite no changes in body weight, whereas blockade of brain *de novo* ceramide synthesis resulted in delayed puberty and obliterated the stimulatory effects of kisspeptins on pubertal maturation. Notably, this phenomenon was conducted via gonadotropin-independent modulation, and involved a previously unnoticed hypothalamic pathway, involving the PVN and the sympathetic innervation of the ovary. In fact, obese pubertal female rats were shown to display changes in kisspeptin innervation of the PVN and increased content in this nucleus of the enzyme, serine palmitoyltransferase long chain base subunit 1 (SPTLC1), responsible for the initial, limiting step in *de novo* ceramide synthesis (97). In fact, virogenetic blockade of SPTLC1 in the PVN of obese female rats largely prevented pubertal acceleration due to overweight. These findings support that ceramide-related pathways are key to central alteration of female puberty in conditions of early obesity.

In addition, functional genomic analyses have evaluated the role of another component of lipid sensing, namely hypothalamic PPARγ signaling, in the control of puberty and reproduction. Thus, female mice engineered to lack PPARγ in mature neurons displayed normal age of pubertal maturation but markers of ovulatory dysfunction, with smaller litters and a reduction in the number of oocytes released per ovulation. In addition, neuron-specific PPARγ KO females displayed alterations in ovarian cycle length and LH levels, as well as hemorrhagic corpora lutea in the ovaries; yet, they were protected from obesity-induced leptin resistance and ovarian cycle irregularities (82). Of interest, neuronal ablation of PPARγ did not alter body weight or glucose/insulin homeostasis in this genetic model. Whether other means of manipulation (e.g., pharmacological) of brain PPARγ signaling may influence reproductive function is yet to be defined. Likewise, whether other lipid sensing mechanisms in the hypothalamus, such as those mediated by FFAR, FA transporters or the BA receptor, TGR5, participate in the control of puberty and reproduction has not been thoroughly addressed and warrant future investigation. Yet, our preliminary observations suggest FFARs, such as GPR84, and PPARγ might participate in the hypothalamic control of puberty, with a variable role depending on the maturational stage and metabolic status. In the same vein, a very recent study has pointed out a putative role of TGR5 in the central regulation of puberty onset in female rats (98). It must be noted that, in addition, PPARγ signaling at the pituitary, the placenta and the ovary has been shown to participate in the control of female reproduction (99), and the use of PPARγ agonists has been proposed in the context of reproductive disorders bound to metabolic alterations, such as polycystic ovary syndrome.

6 Conclusions

In recent years, we have witnessed a substantial expansion of our knowledge of the hypothalamic mechanisms governing whole-body metabolism and reproduction, and particularly of the central pathways responsible for the metabolic control of puberty and fertility. In this context, besides the elucidation of the important reproductive roles of key peripheral hormones, such as leptin, insulin and ghrelin, and central transmitters with pivotal functions in energy homeostasis, recognition of fundamental components of the reproductive brain, such as Kiss1 neurons, sensitive to nutritional and metabolic cues, have paved the way for the integral comprehension of the neuroendocrine mechanisms whereby metabolism and reproduction are tightly connected, and eventually deregulated in adverse conditions.

On top of such neuropeptide framework, it has become evident recently that additional layers of molecular mediators participate in the fine tuning of puberty and reproductive function, and their modulation by metabolic signals. As a clear example, during the last decade it has been disclosed that epigenetic regulatory mechanisms operate to precisely control pubertal maturation and to convey the influence of the nutritional cues on puberty onset (100). In the same vein, the role of different cellular energy sensors, such as AMPK, mTOR and SIRT1, acting on hypothalamic circuits converging on Kiss1 and GnRH neurons, have been shown to participate in the metabolic control of puberty, coupling body nutritional and metabolic status to pubertal maturation and, eventually, fertility. In addition, illuminated by recent evidence pointing out that lipid sensing mechanisms are key components for the hypothalamic regulation of whole-body metabolism (62), the roles of elements of such lipid sensing pathways in the control of reproductive function have begun to be explored recently. These include, but are not restricted to, the roles of hypothalamic ceramides in obesity-induced pubertal acceleration in females, as well as the role of hypothalamic PPARγ in the modulation of the female reproductive axis and its perturbation by obesogenic diets. As important note, it must be stressed that most of our understanding of roles of these energy-sensing mechanisms and lipid mediators in the control of puberty, as key maturational event of the reproductive axis highly sensitive to metabolic cues, derives from studies in female rodents, whereas our knowledge of the eventual function of these signaling

pathways in males remains virtually null. Given that puberty and reproductive function in the male are also sensitive, albeit to a lesser extent, to body energy status, further research is warranted to analyze whether sex differences exist regarding the pathways for the integrative control of energy balance and reproduction; a contention supported by our recent evidence for the female-specific role of hypothalamic ceramide signaling in the control of puberty onset, which is not observed in male rats (97).

In addition, a major challenge that remains to be solved is how the proposed mechanisms for the integral regulation of whole-body metabolism and reproductive function actually integrate within the complex framework defined by the multiple neuronal and glial populations of the hypothalamus. Recent single cell RNA sequencing studies in the mouse hypothalamus, comparing opposite nutritional states (feeding ad-libitum vs. acute fasting), have revealed significant transcriptional changes in AgRP neurons and other cell types, activated during food deprivation (11). Among the differentially-expressed genes, *Zbtb16*, encoding a transcription factor with a relevant role in neurogenesis; *Fam107b*, that codes for a stress response mediator; *Vgf*, a neuropeptide precursor involved in energy homeostasis; and *Sv2c*, a glycoprotein involved in neurotransmitter release, have been highlighted (11). Whether these factors can contribute to the metabolic control of the reproductive axis is yet to be defined, but the *Vgf*-encoded neuropeptide, TLQP-21, has been previously shown to modulate puberty onset and reproductive hormone secretion in rats (101). Importantly, the above RNA-seq studies not only aid in identifying novel and essential components within the different hypothalamic cell populations that control organism functions, including metabolism and reproduction, but also pave the way for comprehensive approaches involving various multi-omics strategies and specific manipulations, targeting different cell types (e.g., cell type-specific viral transfections, as well as chemo- and optogenetic manipulations). These approaches will allow the generation of functional connectivity mappings among different hypothalamic cell types responsible for the integral control of energy balance and reproduction.

Finally, it is anticipated that future efforts in this domain will aim to elucidate the complete set of biological actions and mechanisms of such metabolic and lipid sensing pathways. The resulting knowledge will be instrumental not only to gain deeper insight into the physiological mechanisms governing puberty and fertility, but also to identify novel therapeutic targets for improved management of high prevalent metabolic and reproductive conditions, ranging from pubertal disorders to polycystic ovary syndrome and obesity.

Author contributions

ER: Writing – original draft, Writing – review & editing. ÁA: Writing – original draft, Writing – review & editing. ML: Writing – review & editing. JC: Writing – original draft, Writing – review & editing. MT: Conceptualization, Funding acquisition, Project administration, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Manfredi-Lozano M, Roa J, Tena-Sempere M. Connecting metabolism and gonadal function: Novel central neuropeptide pathways involved in the metabolic control of puberty and fertility. *Front Neuroendocrinol.* (2018) 48:37–49. doi: 10.1016/j.yfrne.2017.07.008

2. Ruiz-Cruz M, Torres-Granados C, Tena-Sempere M, Roa J. Central and peripheral mechanisms involved in the control of GnRH neuronal function by metabolic factors. *Curr Opin Pharmacol.* (2023) 71:102382. doi: 10.1016/j.coph.2023.102382

3. Pivonello R, Menafra D, Riccio E, Garifalos F, Mazzella M, De Angelis C, et al. Metabolic disorders and male hypogonadotropic hypogonadism. *Front Endocrinol (Lausanne).* (2019) 10:345. doi: 10.3389/fendo.2019.00345

4. Manfredi-Lozano M, Roa J, Ruiz-Pino F, Piet R, Garcia-Galiano D, Pineda R, et al. Defining a novel leptin-melanocortin-kisspeptin pathway involved in the metabolic control of puberty. *Mol Metab*. (2016) 5:844–57. doi: 10.1016/j.molmet.2016.08.003

5. Primeaux SD, Harrison-Bernard LM, Barnes MJ. Neurophysiology of the hypothalamus. In: Uwaifo GI, editor. *The Human Hypothalamus. Contemporary Endocrinology*. Humana, Cham (2021).

6. Hill JW, Elias CF. Neuroanatomical framework of the metabolic control of reproduction. *Physiol Rev*. (2018) 98:2349–80. doi: 10.1152/physrev.00033.2017

7. Sobrino V, Avendano MS, Perdices-Lopez C, Jimenez-Puyer M, Tena-Sempere M. Kisspeptins and the neuroendocrine control of reproduction: Recent progress and new frontiers in kisspeptin research. *Front Neuroendocrinol*. (2022) 65:100977. doi: 10.1016/j.yfrne.2021.100977

8. Fong H, Zheng J, Kurrasch D. The structural and functional complexity of the integrative hypothalamus. *Science*. (2023) 382:388–94. doi: 10.1126/science.adh8488

9. Lopez M, Tena-Sempere M. Estrogens and the control of energy homeostasis: a brain perspective. *Trends Endocrinol Metab*. (2015) 26:411–21. doi: 10.1016/j.tem.2015.06.003

10. Miller BH, Takahashi JS. Central circadian control of female reproductive function. *Front Endocrinol (Lausanne)*. (2013) 4:195. doi: 10.3389/fendo.2013.00195

11. Steuernagel L, Lam BYH, Klemm P, Dowsett GKC, Bauder CA, Tadross JA, et al. HypoMap-a unified single-cell gene expression atlas of the murine hypothalamus. *Nat Metab*. (2022) 4:1402–19. doi: 10.1038/s42255-022-00657-y

12. Clasadonte J, Prevot V. The special relationship: glia-neuron interactions in the neuroendocrine hypothalamus. *Nat Rev Endocrinol*. (2018) 14:25–44. doi: 10.1038/nrendo.2017.124

13. Nampoothiri S, Nogueiras R, Schwaninger M, Prevot V. Glial cells as integrators of peripheral and central signals in the regulation of energy homeostasis. *Nat Metab*. (2022) 4:813–25. doi: 10.1038/s42255-022-00610-z

14. Torres E, Pellegrino G, Granados-Rodriguez M, Fuentes-Fayos AC, Velasco I, Coutteau-Robles A, et al. Kisspeptin signaling in astrocytes modulates the reproductive axis. *J Clin Invest*. (2024) 134:e172908. doi: 10.1172/JCI172908

15. Sung Y, Yu YC, Han JM. Nutrient sensors and their crosstalk. *Exp Mol Med*. (2023) 55:1076–89. doi: 10.1038/s12276-023-01006-z

16. Templeman NM, Murphy CT. Regulation of reproduction and longevity by nutrient-sensing pathways. *J Cell Biol*. (2018) 217:93–106. doi: 10.1083/jcb.201707168

17. Lopez M, Nogueiras R, Tena-Sempere M, Dieguez C. Hypothalamic AMPK: a canonical regulator of whole-body energy balance. *Nat Rev Endocrinol*. (2016) 12:421–32. doi: 10.1038/nrendo.2016.67

18. Efeyan A, Comb WC, Sabatini DM. Nutrient-sensing mechanisms and pathways. *Nature*. (2015) 517:302–10. doi: 10.1038/nature14190

19. Steinberg GR, Hardie DG. New insights into activation and function of the AMPK. *Nat Rev Mol Cell Biol*. (2023) 24:255–72. doi: 10.1038/s41580-022-00547-x

20. Grahame Hardie D. AMP-activated protein kinase: a key regulator of energy balance with many roles in human disease. *J Intern Med*. (2014) 276:543–59. doi: 10.1111/joim.12268

21. Lopez M. Hypothalamic AMPK as a possible target for energy balance-related diseases. *Trends Pharmacol Sci*. (2022) 43:546–56. doi: 10.1016/j.tips.2022.04.007

22. McCrimmon RJ, Shaw M, Fan X, Cheng H, Ding Y, Vella MC, et al. Key role for AMP-activated protein kinase in the ventromedial hypothalamus in regulating counterregulatory hormone responses to acute hypoglycemia. *Diabetes*. (2008) 57:444–50. doi: 10.2337/db07-0837

23. Martinez-Sanchez N, Seoane-Collazo P, Contreras C, Varela L, Villarroya J, Rial-Pensado E, et al. Hypothalamic AMPK-ER stress-JNK1 axis mediates the central actions of thyroid hormones on energy balance. *Cell Metab*. (2017) 26:212–229.e12. doi: 10.1016/j.cmet.2017.06.014

24. Claret M, Smith MA, Batterham RL, Selman C, Choudhury AI, Fryer LG, et al. AMPK is essential for energy homeostasis regulation and glucose sensing by POMC and AgRP neurons. *J Clin Invest*. (2007) 117:2325–36. doi: 10.1172/JCI31516

25. Seoane-Collazo P, Roa J, Rial-Pensado E, Linares-Pose L, Beiroa D, Ruiz-Pino F, et al. SF1-specific AMPKalpha1 deletion protects against diet-induced obesity. *Diabetes*. (2018) 67:2213–26. doi: 10.2337/db17-1538

26. Pena-Leon V, Perez-Lois R, Seoane LM. mTOR pathway is involved in energy homeostasis regulation as a part of the gut-brain axis. *Int J Mol Sci*. (2020) 21:5715. doi: 10.3390/ijms21165715

27. Kim SG, Buel GR, Blenis J. Nutrient regulation of the mTOR complex 1 signaling pathway. *Mol Cells*. (2013) 35:463–73. doi: 10.1007/s10059-013-0138-2

28. Gonzalez A, Hall MN, Lin SC, Hardie DG. AMPK and TOR: the yin and yang of cellular nutrient sensing and growth control. *Cell Metab*. (2020) 31:472–92. doi: 10.1016/j.cmet.2020.01.015

29. Cota D, Proulx K, Smith KA, Kozma SC, Thomas G, Woods SC, et al. Hypothalamic mTOR signaling regulates food intake. *Science*. (2006) 312:927–30. doi: 10.1126/science.1124147

30. Martins L, Fernandez-Mallo D, Novelle MG, Vazquez MJ, Tena-Sempere M, Nogueiras R, et al. Hypothalamic mTOR signaling mediates the orexigenic action of ghrelin. *PloS One*. (2012) 7:e46923. doi: 10.1371/journal.pone.0046923

31. Martinez De Morentin PB, Martinez-Sanchez N, Roa J, Ferno J, Nogueiras R, Tena-Sempere M, et al. Hypothalamic mTOR: the rookie energy sensor. *Curr Mol Med*. (2014) 14:3–21. doi: 10.2174/1566524013666131118103706

32. Szwed A, Kim E, Jacinto E. Regulation and metabolic functions of mTORC1 and mTORC2. *Physiol Rev*. (2021) 101:1371–426. doi: 10.1152/physrev.00026.2020

33. Villanueva EC, Munzberg H, Cota D, Leshan RL, Kopp K, Ishida-Takahashi R, et al. Complex regulation of mammalian target of rapamycin complex 1 in the basomedial hypothalamus by leptin and nutritional status. *Endocrinology*. (2009) 150:4541–51. doi: 10.1210/en.2009-0642

34. Zhang W, Zhang C, Fritze D, Chai B, Li J, Mulholland MW. Modulation of food intake by mTOR signalling in the dorsal motor nucleus of the vagus in male rats: focus on ghrelin and nesfatin-1. *Exp Physiol*. (2013) 98:1696–704. doi: 10.1113/expphysiol.2013.074930

35. Yuan Y, Cruzat VF, Newsholme P, Cheng J, Chen Y, Lu Y. Regulation of SIRT1 in aging: Roles in mitochondrial function and biogenesis. *Mech Ageing Dev*. (2016) 155:10–21. doi: 10.1016/j.mad.2016.02.003

36. Nogueiras R, Habegger KM, Chaudhary N, Finan B, Banks AS, Dietrich MO, et al. Sirtuin 1 and sirtuin 3: physiological modulators of metabolism. *Physiol Rev*. (2012) 92:1479–514. doi: 10.1152/physrev.00022.2011

37. Imai S, Armstrong CM, Kaeberlein M, Guarente L. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature*. (2000) 403:795–800. doi: 10.1038/35001622

38. Quinones M, Martinez-Grobas E, Ferno J, Perez-Lois R, Seoane LM, Al Massadi O. Hypothalamic actions of SIRT1 and SIRT6 on energy balance. *Int J Mol Sci*. (2021) 22:1430. doi: 10.3390/ijms22031430

39. Ramadori G, Lee CE, Bookout AL, Lee S, Williams KW, Anderson J, et al. Brain SIRT1: anatomical distribution and regulation by energy availability. *J Neurosci*. (2008) 28:9989–96. doi: 10.1523/JNEUROSCI.3257-08.2008

40. Sasaki T, Kitamura T. Roles of FoxO1 and Sirt1 in the central regulation of food intake. *Endocr J*. (2010) 57:939–46. doi: 10.1507/endocrj.K10E-320

41. Velasquez DA, Martinez G, Romero A, Vazquez MJ, Boit KD, Dopeso-Reyes IG, et al. The central Sirtuin 1/p53 pathway is essential for the orexigenic action of ghrelin. *Diabetes*. (2011) 60:1177–85. doi: 10.2337/db10-0802

42. Ramadori G, Fujikawa T, Fukuda M, Anderson J, Morgan DA, Mostoslavsky R, et al. SIRT1 deacetylase in POMC neurons is required for homeostatic defenses against diet-induced obesity. *Cell Metab*. (2010) 12:78–87. doi: 10.1016/j.cmet.2010.05.010

43. Ruderman NB, Xu XJ, Nelson L, Cacicedo JM, Saha AK, Lan F, et al. AMPK and SIRT1: a long-standing partnership? *Am J Physiol Endocrinol Metab*. (2010) 298:E751–60. doi: 10.1152/ajpendo.00745.2009

44. Ramadori G, Fujikawa T, Anderson J, Berglund ED, Frazao R, Michan S, et al. SIRT1 deacetylase in SF1 neurons protects against metabolic imbalance. *Cell Metab*. (2011) 14:301–12. doi: 10.1016/j.cmet.2011.06.014

45. Tang Q, Gao Y, Liu Q, Yang X, Wu T, Huang C, et al. Sirt6 in pro-opiomelanocortin neurons controls energy metabolism by modulating leptin signaling. *Mol Metab*. (2020) 37:100994. doi: 10.1016/j.molmet.2020.100994

46. Quinones M, Hernandez-Bautista R, Beiroa D, Heras V, Torres-Leal FL, Lam BYH, et al. Sirt3 in POMC neurons controls energy balance in a sex- and diet-dependent manner. *Redox Biol*. (2021) 41:101945. doi: 10.1016/j.redox.2021.101945

47. Coyral-Castel S, Tosca L, Ferreira G, Jeanpierre E, Rame C, Lomet D, et al. The effect of AMP-activated kinase activation on gonadotrophin-releasing hormone secretion in GT1-7 cells and its potential role in hypothalamic regulation of the oestrous cyclicity in rats. *J Neuroendocrinol*. (2008) 20:335–46. doi: 10.1111/j.1365-2826.2007.01643.x

48. Roa J, Barroso A, Ruiz-Pino F, Vazquez MJ, Seoane-Collazo P, Martinez-Sanchez N, et al. Metabolic regulation of female puberty via hypothalamic AMPK-kisspeptin signaling. *Proc Natl Acad Sci USA*. (2018) 115:E10758–67. doi: 10.1073/pnas.1802053115

49. Wen JP, Liu C, Bi WK, Hu YT, Chen Q, Huang H, et al. Adiponectin inhibits KISS1 gene transcription through AMPK and specificity protein-1 in the hypothalamic GT1-7 neurons. *J Endocrinol*. (2012) 214:177–89. doi: 10.1530/JOE-12-0054

50. Torsoni MA, Borges BC, Cote JL, Allen SJ, Mahany E, Garcia-Galiano D, et al. AMPKalpha2 in kiss1 neurons is required for reproductive adaptations to acute metabolic challenges in adult female mice. *Endocrinology*. (2016) 157:4803–16. doi: 10.1210/en.2016-1367

51. Franssen D, Barroso A, Ruiz-Pino F, Vazquez MJ, Garcia-Galiano D, Castellano JM, et al. AMP-activated protein kinase (AMPK) signaling in GnRH neurons links energy status and reproduction. *Metabolism*. (2021) 115:154460. doi: 10.1016/j.metabol.2020.154460

52. Perdices-Lopez C, Avendano MS, Barroso A, Gaytan F, Ruiz-Pino F, Vazquez MJ, et al. Connecting nutritional deprivation and pubertal inhibition via GRK2-mediated repression of kisspeptin actions in GnRH neurons. *Metabolism*. (2022) 129:155141. doi: 10.1016/j.metabol.2022.155141

53. Roa J, Tena-Sempere M. Energy balance and puberty onset: emerging role of central mTOR signaling. *Trends Endocrinol Metab*. (2010) 21:519–28. doi: 10.1016/j.tem.2010.05.003

54. Roa J, Garcia-Galiano D, Varela L, Sanchez-Garrido MA, Pineda R, Castellano JM, et al. The mammalian target of rapamycin as novel central regulator of puberty onset via modulation of hypothalamic Kiss1 system. *Endocrinology*. (2009) 150:5016–26. doi: 10.1210/en.2009-0096

55. Beymer M, Negron AL, Yu G, Wu S, Mayer C, Lin RZ, et al. Kisspeptin cell-specific PI3K signaling regulates hypothalamic kisspeptin expression and participates

in the regulation of female fertility. *Am J Physiol Endocrinol Metab*. (2014) 307:E969–82. doi: 10.1152/ajpendo.00385.2014

56. Negron AL, Yu G, Boehm U, Acosta-Martinez M. Targeted deletion of PTEN in kisspeptin cells results in brain region- and sex-specific effects on kisspeptin expression and gonadotropin release. *Int J Mol Sci*. (2020) 21:2107. doi: 10.3390/ijms21062107

57. Di Sante G, Wang L, Wang C, Jiao X, Casimiro MC, Chen K, et al. Sirt1-deficient mice have hypogonadotropic hypogonadism due to defective GnRH neuronal migration. *Mol Endocrinol*. (2015) 29:200–12. doi: 10.1210/me.2014-1228

58. McBurney MW, Yang X, Jardine K, Hixon M, Boekelheide K, Webb JR, et al. The mammalian SIR2alpha protein has a role in embryogenesis and gametogenesis. *Mol Cell Biol*. (2003) 23:38–54. doi: 10.1128/MCB.23.1.38-54.2003

59. Kolthur-Seetharam U, Teerds K, De Rooij DG, Wendling O, Mcburney M, Sassone-Corsi P, et al. The histone deacetylase SIRT1 controls male fertility in mice through regulation of hypothalamic-pituitary gonadotropin signaling. *Biol Reprod*. (2009) 80:384–91. doi: 10.1095/biolreprod.108.070193

60. Vazquez MJ, Toro CA, Castellano JM, Ruiz-Pino F, Roa J, Beiroa D, et al. SIRT1 mediates obesity- and nutrient-dependent perturbation of pubertal timing by epigenetically controlling Kiss1 expression. *Nat Commun*. (2018) 9:4194. doi: 10.1038/s41467-018-06459-9

61. Choi I, Rickert E, Fernandez M, Webster NJG. SIRT1 in astrocytes regulates glucose metabolism and reproductive function. *Endocrinology*. (2019) 160:1547–60. doi: 10.1210/en.2019-00223

62. Dragano NR, Monfort-Pires M, Velloso LA. Mechanisms mediating the actions of fatty acids in the hypothalamus. *Neuroscience*. (2020) 447:15–27. doi: 10.1016/j.neuroscience.2019.10.012

63. Bruce KD, Zsombok A, Eckel RH. Lipid processing in the brain: A key regulator of systemic metabolism. *Front Endocrinol (Lausanne)*. (2017) 8:60. doi: 10.3389/fendo.2017.00060

64. Falomir-Lockhart LJ, Cavazzutti GF, Gimenez E, Toscani AM. Fatty acid signaling mechanisms in neural cells: fatty acid receptors. *Front Cell Neurosci*. (2019) 13:162. doi: 10.3389/fncel.2019.00162

65. Kimura I, Ichimura A, Ohue-Kitano R, Igarashi M. Free fatty acid receptors in health and disease. *Physiol Rev*. (2020) 100:171–210. doi: 10.1152/physrev.00041.2018

66. Samovski D, Jacome-Sosa M, Abumrad NA. Fatty acid transport and signaling: mechanisms and physiological implications. *Annu Rev Physiol*. (2023) 85:317–37. doi: 10.1146/annurev-physiol-032122-030352

67. Fosch A, Rodriguez-Garcia M, Miralpeix C, Zagmutt S, Larranaga M, Reguera AC, et al. Central regulation of brown fat thermogenesis in response to saturated or unsaturated long-chain fatty acids. *Int J Mol Sci*. (2023) 24:1697. doi: 10.3390/ijms24021697

68. Auguste S, Fisette A, Fernandes MF, Hryhorczuk C, Poitout V, Alquier T, et al. Central agonism of GPR120 acutely inhibits food intake and food reward and chronically suppresses anxiety-like behavior in mice. *Int J Neuropsychopharmacol*. (2016) 19:1–10. doi: 10.1093/ijnp/pyw014

69. Wellhauser L, Belsham DD. Activation of the omega-3 fatty acid receptor GPR120 mediates anti-inflammatory actions in immortalized hypothalamic neurons. *J Neuroinflamm*. (2014) 11:60. doi: 10.1186/1742-2094-11-60

70. Dragano NRV, Milbank E, Haddad-Tovolli R, Garrido-Gil P, Novoa E, Fondevilla MF, et al. Hypothalamic free fatty acid receptor-1 regulates whole-body energy balance. *Mol Metab*. (2024) 79:101840. doi: 10.1016/j.molmet.2023.101840

71. Velasco C, Conde-Sieira M, Comesana S, Chivite M, Miguez JM, Soengas JL. Role of the G protein-coupled receptors GPR84 and GPR119 in the central regulation of food intake in rainbow trout. *J Exp Biol*. (2021) 224:jeb242360. doi: 10.1242/jeb.242360

72. Zhang D, Jian YP, Zhang YN, Li Y, Gu LT, Sun HH, et al. Short-chain fatty acids in diseases. *Cell Commun Signal*. (2023) 21:212. doi: 10.1186/s12964-023-01219-9

73. Loona DPS, Das B, Kaur R, Kumar R, Yadav AK. Free fatty acid receptors (FFARs): emerging therapeutic targets for the management of diabetes mellitus. *Curr Med Chem*. (2023) 30:3404–40. doi: 10.2174/0929867329666220927113614

74. Mishra SP, Karunakar P, Taraphder S, Yadav H. Free fatty acid receptors 2 and 3 as microbial metabolite sensors to shape host health: pharmacophysiological view. *Biomedicines*. (2020) 8:154. doi: 10.3390/biomedicines8060154

75. Chao YM, Tain YL, Lee WC, Wu KLH, Yu HR, Chan JYH. Protection by -biotics against hypertension programmed by maternal high fructose diet: rectification of dysregulated expression of short-chain fatty acid receptors in the hypothalamic paraventricular nucleus of adult offspring. *Nutrients*. (2022) 14:4306. doi: 10.3390/nu14204306

76. Le Foll C. Hypothalamic fatty acids and ketone bodies sensing and role of FAT/CD36 in the regulation of food intake. *Front Physiol*. (2019) 10:1036. doi: 10.3389/fphys.2019.01036

77. Hao JW, Wang J, Guo H, Zhao YY, Sun HH, Li YF, et al. CD36 facilitates fatty acid uptake by dynamic palmitoylation-regulated endocytosis. *Nat Commun*. (2020) 11:4765. doi: 10.1038/s41467-020-18565-8

78. Le Foll C, Dunn-Meynell A, Musatov S, Magnan C, Levin BE. FAT/CD36: a major regulator of neuronal fatty acid sensing and energy homeostasis in rats and mice. *Diabetes*. (2013) 62:2709–16. doi: 10.2337/db12-1689

79. Ochiai Y, Uchida Y, Ohtsuki S, Tachikawa M, Aizawa S, Terasaki T. The blood-brain barrier fatty acid transport protein 1 (FATP1/SLC27A1) supplies docosahexaenoic acid to the brain, and insulin facilitates transport. *J Neurochem*. (2017) 141:400–12. doi: 10.1111/jnc.13943

80. Mitchell RW, On NH, Del Bigio MR, Miller DW, Hatch GM. Fatty acid transport protein expression in human brain and potential role in fatty acid transport across human brain microvessel endothelial cells. *J Neurochem*. (2011) 117:735–46. doi: 10.1111/j.1471-4159.2011.07245.x

81. Dixon ED, Nardo AD, Claudel T, Trauner M. The role of lipid sensing nuclear receptors (PPARs and LXR) and metabolic lipases in obesity, diabetes and NAFLD. *Genes (Basel)*. (2021) 12:645. doi: 10.3390/genes12050645

82. Fernandez MO, Sharma S, Kim S, Rickert E, Hsueh K, Hwang V, et al. Obese neuronal PPARgamma knockout mice are leptin sensitive but show impaired glucose tolerance and fertility. *Endocrinology*. (2017) 158:121–33. doi: 10.1210/en.2016-1818

83. Garretson JT, Teubner BJ, Grove KL, Vazdarjanova A, Ryu V, Bartness TJ. Peroxisome proliferator-activated receptor gamma controls ingestive behavior, agouti-related protein, and neuropeptide Y mRNA in the arcuate hypothalamus. *J Neurosci*. (2015) 35:4571–81. doi: 10.1523/JNEUROSCI.2129-14.2015

84. Li Q, Yu Q, Lin L, Zhang H, Peng M, Jing C, et al. Hypothalamic peroxisome proliferator-activated receptor gamma regulates ghrelin production and food intake. *Neuropeptides*. (2018) 69:39–45. doi: 10.1016/j.npep.2018.04.002

85. Long L, Toda C, Jeong JK, Horvath TL, Diano S. PPARgamma ablation sensitizes proopiomelanocortin neurons to leptin during high-fat feeding. *J Clin Invest*. (2014) 124:4017–27. doi: 10.1172/JCI76220

86. Chaurasia B, Summers SA. Ceramides in metabolism: key lipotoxic players. *Annu Rev Physiol*. (2021) 83:303–30. doi: 10.1146/annurev-physiol-031620-093815

87. Gonzalez-Garcia I, Contreras C, Estevez-Salguero A, Ruiz-Pino F, Colsh B, Pensado I, et al. Estradiol regulates energy balance by ameliorating hypothalamic ceramide-induced ER stress. *Cell Rep*. (2018) 25:413–423.e5. doi: 10.1016/j.celrep.2018.09.038

88. Contreras C, Gonzalez-Garcia I, Martinez-Sanchez N, Seoane-Collazo P, Jacas J, Morgan DA, et al. Central ceramide-induced hypothalamic lipotoxicity and ER stress regulate energy balance. *Cell Rep*. (2014) 9:366–77. doi: 10.1016/j.celrep.2014.08.057

89. Campana M, Bellini L, Rouch C, Rachdi L, Coant N, Butin N, et al. Inhibition of central *de novo* ceramide synthesis restores insulin signaling in hypothalamus and enhances beta-cell function of obese Zucker rats. *Mol Metab*. (2018) 8:23–36. doi: 10.1016/j.molmet.2017.10.013

90. Hammerschmidt P, Steculorum SM, Bandet CL, Del Rio-Martin A, Steuernagel L, Kohlhaas V, et al. CerS6-dependent ceramide synthesis in hypothalamic neurons promotes ER/mitochondrial stress and impairs glucose homeostasis in obese mice. *Nat Commun*. (2023) 14:7824. doi: 10.1038/s41467-023-42595-7

91. Teo JD, Marian OC, Spiteri AG, Nicholson M, Song H, Khor JXY, et al. Early microglial response, myelin deterioration and lethality in mice deficient for very long chain ceramide synthesis in oligodendrocytes. *Glia*. (2023) 71:1120–41. doi: 10.1002/glia.24329

92. Xing C, Huang X, Wang D, Yu D, Hou S, Cui H, et al. Roles of bile acids signaling in neuromodulation under physiological and pathological conditions. *Cell Biosci*. (2023) 13:106. doi: 10.1186/s13578-023-01053-z

93. Hylemon PB, Zhou H, Pandak WM, Ren S, Gil G, Dent P. Bile acids as regulatory molecules. *J Lipid Res*. (2009) 50:1509–20. doi: 10.1194/jlr.R900007-JLR200

94. Higashi T, Watanabe S, Tomaru K, Yamazaki W, Yoshizawa K, Ogawa S, et al. Unconjugated bile acids in rat brain: Analytical method based on LC/ESI-MS/MS with chemical derivatization and estimation of their origin by comparison to serum levels. *Steroids*. (2017) 125:107–13. doi: 10.1016/j.steroids.2017.07.001

95. Castellanos-Jankiewicz A, Guzman-Quevedo O, Fenelon VS, Zizzari P, Quarta C, Bellocchio L, et al. Hypothalamic bile acid-TGR5 signaling protects from obesity. *Cell Metab*. (2021) 33:1483–1492.e10. doi: 10.1016/j.cmet.2021.04.009

96. Perino A, Velazquez-Villegas LA, Bresciani N, Sun Y, Huang Q, Fenelon VS, et al. Central anorexigenic actions of bile acids are mediated by TGR5. *Nat Metab*. (2021) 3:595–603. doi: 10.1038/s42255-021-00398-4

97. Heras V, Castellano JM, Fernandois D, Velasco I, Rodriguez-Vazquez E, Roa J, et al. Central ceramide signaling mediates obesity-induced precocious puberty. *Cell Metab*. (2020) 32:951–966.e8. doi: 10.1016/j.cmet.2020.10.001

98. Vanden Brink H, Vandeputte D, Brito IL, Ronnekleiv OK, Roberson MS, Lomniczi A. Changes in the bile acid pool and timing of female puberty: potential novel role of hypothalamic TGR5. *Endocrinology*. (2024). doi: 10.1210/endocr/bqae098

99. Froment P, Gizard F, Defever D, Staels B, Dupont J, Monget P. Peroxisome proliferator-activated receptors in reproductive tissues: from gametogenesis to parturition. *J Endocrinol*. (2006) 189:199–209. doi: 10.1677/joe.1.06667

100. Argente J, Dunkel L, Kaiser UB, Latronico AC, Lomniczi A, Soriano-Guillen L, et al. Molecular basis of normal and pathological puberty: from basic mechanisms to clinical implications. *Lancet Diabetes Endocrinol*. (2023) 11:203–16. doi: 10.1016/S2213-8587(22)00339-4

101. Aguilar E, Pineda R, Gaytan F, Sanchez-Garrido MA, Romero M, Romero-Ruiz A, et al. Characterization of the reproductive effects of the Vgf-derived peptide TLQP-21 in female rats: in *vivo* and in *vitro* studies. *Neuroendocrinology*. (2013) 98:38–50. doi: 10.1159/000350323

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Challenges in molecular diagnosis of multiple endocrine neoplasia

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Multiple endocrine neoplasia (MEN) is a group of rare genetic diseases characterized by the occurrence of multiple tumors of the endocrine system in the same patient. The first MEN described was MEN1, followed by MEN2A, and MEN2B. The identification of the genes responsible for these syndromes led to the introduction of family genetic screening programs. More than twenty years later, not all cases of MENs have been resolved from a genetic point of view, and new clinicogenetic entities have been described. In this review, we will discuss the strategies and difficulties of genetic screening for classic and newly described MENs in a clinical setting, from limitations in sequencing, to problems in classifying variants, to the identification of new candidate genes. In the era of genomic medicine, characterization of new candidate genes and their specific tumor risk is essential for inclusion of patients in personalized medicine programs as well as to permit accurate genetic counseling to be proposed for families.

KEYWORDS

MEN1, MEN2, genetic testing, candidate gene, genome, mosaicism

1 Introduction

Multiple endocrine neoplasia (MEN) is a group of diseases characterized by the occurrence of multiple tumors of the endocrine system in the same patient. MENs are rare genetic diseases resulting from germline genetic defects in a variety of tumor suppressor genes or oncogenes. MENs are hereditary diseases, transmitted in an autosomal dominant manner. The first MEN described was MEN1 (OMIM 131100) (1), followed by MEN2A (OMIM 171400) and MEN2B (OMIM 162300). Initially these syndromes were described clinically, followed by the discovery of underlying genetic causes. Identification of these disease-specific genes has enabled targeted genetic testing of index cases, and in the event of positive results, presymptomatic screening of their relatives. Presymptomatic genetic testing is a crucial aspect of personalized medicine in tumor predisposition syndromes as it enables the identification of carriers and non-carriers of pathogenic or likely pathogenic variants in a family before they show any clinical signs or

symptoms of the condition (2). Family members who carry such variants can then benefit from a disease-specific monitoring program, while non-carriers can be reassured and excluded from monitoring. However, a genetic basis is not identified in all MEN patients. More recently, advances in medicine, scientific knowledge and sequencing technologies have revolutionized our conception of MENs, enabling the discovery of new entities such as MEN4 (OMIM 610755) (3) and the resolution of failures in molecular diagnosis. With advances in genomic sequencing technology, the number of gene-disease relationships that have been described has rapidly expanded, though their application in clinical settings is sometimes uncertain.

2 MEN syndromes

2.1 MEN1

Multiple endocrine neoplasia type 1 results from an inactivating heterozygous mutation in the *MEN1* tumor suppressor gene (Table 1) (1). *MEN1* (location 11q13.1) encodes MENIN, a nuclear scaffold protein that is involved in histone modification and epigenetic gene regulation (4). Indeed, the MENIN is an essential component of the MLL/SET1 histone methyltransferase (HMT) complex, a complex that specifically methylates 'Lys-4' of histone H3 (H3K4) (5, 6). H3K4 trimethylation is associated with activated gene transcription. A loss of MENIN results in transcriptional repression of specific genes due to loss of H3K4me3 in the promoter. MENIN also functions as a transcriptional regulator, binding to the *TERT* promoter and repressing telomerase expression. It interacts with SMAD3 or SMAD1/SMAD5 to promote their transcriptional activity, and the loss of MENIN in these interactions inhibits the TGF-β and BMP signaling pathways, respectively, thus antagonizing their proliferation-inhibitory action (7). MENIN also represses JUND-mediated transcriptional activation (8). JUND is a member of the JUN family, and a functional component of the AP1 transcription factor complex. It has been proposed that JUND protects cells from p53-dependent senescence and apoptosis. MENIN positively regulates *HOXC8* and *HOXC6* expression and may be involved in normal hematopoiesis through the activation of *HOXA9* expression. In MEN1, patients are predisposed to develop tumors by inheritance of a heterozygous inactivating mutation, while it is complete loss of MENIN or inactivation by a somatic second hit on the other allele that promotes tumorigenesis, according to the two hit Knudson hypothesis (9). MEN1 prevalence is estimated at between 1/10,000 and 1/30,000. The classic clinical triad observed in MEN1 includes primary hyperparathyroidism (PHPT), pituitary neuroendocrine tumors (PitNETs), and duodeno-pancreatic neuroendocrine tumors (DPNETs). Other endocrine tumors, including adrenal cortical tumors, and neuroendocrine thymic or bronchopulmonary tumors, may also be present. Several non-endocrine manifestations have also been reported to be associated with MEN1: angiofibromas, collagenomas, lipomas, and meningiomas. Twenty-eight to 70% of patients with MEN1 die as a consequence of the disease, particularly due to the grade neuroendocrine tumor (NET) lesions (10, 11). MEN1 diagnosis is classically made where any of three different criteria are met: i) the presence of 2 MEN1-related major lesion in one patient, ii) the presence of MEN1-related lesion in a patient with a first degree relative with MEN1, iii) the presence of a MEN1 pathogenic or likely pathogenic variant in a patient, which may or may not be symptomatic (12). An important effort has been made by the international research community to provide clinical practice guidelines for monitoring and genetic testing, and these are currently under revision (for most recent version see (12)).

2.2 MEN2

Multiple endocrine neoplasia type 2 occurs due to recurrent heterozygous activating mutations in the proto-oncogene *RET* (Table 1). *RET* (location 10q11.21) encodes RET, a transmembrane receptor and member of the tyrosine protein kinase family of proteins. Binding of ligands such as GDNF (glial cell-line derived neurotrophic factor) and other related proteins to the encoded receptor stimulates receptor dimerization, RET intracellular transphosphorylation, and activation of downstream signaling pathways that play a role in cell differentiation, growth, migration and survival. MEN2 prevalence is estimated at

TABLE 1 Multiple endocrine neoplasia syndromes.

Syndrome	Gene	Gene type	Transmission	Phenotype
MEN1	*MEN1*	tumor suppressor	autosomal dominant	Primary hyperparathyroidism, pituitary NET, duodeno-pancreatic NET, lung NET, thymic NET, adrenal tumor, lipomas, angiofibromas, collagenomas, hibernomas, leiomyomas, central nervous system tumors, breast cancer
MEN2A	*RET*	oncogene	autosomal dominant	Medullary thyroid carcinoma, pheochromocytoma, primary hyperparathyroidism
MEN2B	*RET*	oncogene	autosomal dominant	Early onset medullary thyroid carcinoma, pheochromocytoma, intestinal ganglioneuromatosis, Marfanoid habitus, alacryma, mucosal neuromas
MEN4	*CDKN1B*	tumor suppressor gene	autosomal dominant	Primary hyperparathyroidism, pituitary NET, duodeno-pancreatic NET, adrenal tumor, thymus tumor, papillary thyroid cancer

NET, neuroendocrine tumor.

approximately 1/35,000. MEN2 was initially separated into three syndromes: MEN2A (95% of MEN2), MEN2B (also called MEN3), and familial medullary thyroid carcinoma (FMTC), due to very specific clinical presentations that depend on the mutations present. Indeed, MEN2 is characterized by a strong genotype-phenotype correlation (13). Major clinical manifestations of MEN2 include medullary thyroid carcinoma (MTC), pheochromocytoma (PHEO) and, in the case of MEN2A, primary hyperparathyroidism (pHPT). The term FMTC has been gradually abandoned in favor of MEN2A, with the identification of low-frequency lesions associated with MTC. Here too, an important effort has been made to provide clinical practice guidelines for monitoring and genetic counseling (13, 14).

MEN2A is mainly caused by activating mutations in exons 10 and 11, and is characterized by variable risks of aggressive MTC, pheochromocytoma, PHPT or cutaneous lichen amyloid depending on the mutation (14). Mutations in exons 10 and 11 affect cysteines in the extracellular domain of the RET receptor. They cause dimerization of receptor molecules, enhanced phosphorylation and thus ligand-independent activation of intra-cellular pathways. Mutations in codon Cys634 (exon 11) are associated with a high risk of aggressive MTC as well as a very high risk of pheochromocytoma (about 50%) and, to a lesser extent, of PHPT. MEN2A *RET* mutations in exon 10 can be associated with Hirschsprung's disease, a rare congenital intestinal motility disorder, due to the presence of an aganglionic segment in the terminal part of the colon. MEN2A variants in the intracellular tyrosine kinase domain are less frequent (exons 13 to 16). These are associated with a milder risk of PHTP and pheochromocytoma, except for the M918T variant in exon 19 and the A883F variant in exon 15 that cause MEN2B.

MEN2B is mainly due to a specific activating mutation c.2753T>C, p.Met918Thr (M918T). This change from a methionine to threonine within the activation segment of RET kinase, increases ATP-binding and auto-phosphorylation activity, thereby mediating a dimerization-independent activation of RET kinase. MEN2B is characterized by early and aggressive medullary thyroid carcinoma (MTC), pheochromocytoma, mucosal neuromas, and thickened corneal nerves. Most affected individuals have characteristic physical features, including full lips, thickened eyelids, high-arched palate, and marfanoid habitus (long arms, long legs, arachnodactyly). Less frequently affected individuals present skeletal anomalies and gastrointestinal problems (15).

2.3 MEN4

Multiple endocrine neoplasia type 4 (MEN4) results from heterozygous inactivating mutations in the *CDKN1B* tumor suppressor gene (Table 1) (3). *CDKN1B* (location 12p13) encodes P27KIP1 which is a cyclin-dependent kinase inhibitor that is normally activated by MENIN (encoded by *MEN1*) to negatively regulate the cell cycle and limit G1-S phase transition. MEN4 was identified in human by its homology with a mouse model that develops MEN1-related lesions. The prevalence of MEN4 is unknown, but is lower than MEN1, with MEN4 patients developing a MEN1-like phenotype with a later onset and with incomplete penetrance (3, 16). To date, there is no consensus on genetic testing, monitoring, and genetic counseling for MEN4.

3 MENs -diagnostic challenges and pitfalls

3.1 General strategy for molecular diagnosis of MENs:

In practice, patients with clinical or suspected MEN may benefit from germline genetic testing targeting the disease-specific gene. Traditionally, genetic tests in index cases targeted a single gene and were performed using the Sanger method. The Sanger method is a DNA sequencing method based on the random incorporation of chain-terminating fluorescent dideoxynucleotides by DNA polymerase during *in vitro* DNA replication, that are detected by electrophoresis (17). More recently, the Sanger method was abandoned in favor of new methods that enable several genes to be examined at the same time, known as next generation sequencing (NGS) procedures (18). NGS platforms perform sequencing of millions of small fragments of DNA, called reads, in parallel. NGS rapidly became preferred over the Sanger method for genetic testing of index cases because i) NGS captures a broader spectrum of mutations than Sanger sequencing, from small base changes (substitutions) to large genomic deletions of exons or whole genes (which Sanger sequencing cannot do); ii) NGS is cost effective, allowing the sequencing of several genes, up to the whole genome, in several samples, simultaneously.

After sequencing, variants are classified into 5 classes of pathogenicity: class 1: benign variant (BV), class 2: likely benign variant (LBV), class 3: variant of uncertain significance (VUS), class 4: likely pathogenic variant (LPV), class 5: pathogenic variant (PV, see Figure 1 (19). The use of this classification system allows medical care to be adapted according to the likely effect of the variation. For a patient with a disease, a BV or a LBV in a gene is considered as not causing or likely to not cause the disease, while a PV is considered to be responsible for the disease. In the case of a LPV, this means that there is enough evidence to consider that the variant causes the disease, but there is still a certain degree of uncertainty. Consequently, this information should be used with caution for clinical decision-making. In the case of PV or LPV, the test is considered as positive and the diagnosis is confirmed, even in patients with an incomplete phenotype. Moreover, their relatives can benefit from genetic counseling, including presymptomatic screening. Pre-symptomatic screening is based on a targeted search of the familial anomaly, generally by Sanger sequencing looking for single nucleotide variations (SNV) and MLPA for copy number variations (CNV).

The next category, VUS, consists of variants suspected of causing the disease, but with insufficient or conflicting evidence. The distinction between LPV and VUS is very important because the presence of a LPV can be used not only for decision making regarding patient care, but also for genetic counseling, including the testing of family members, prenatal diagnosis etc (20). On the other

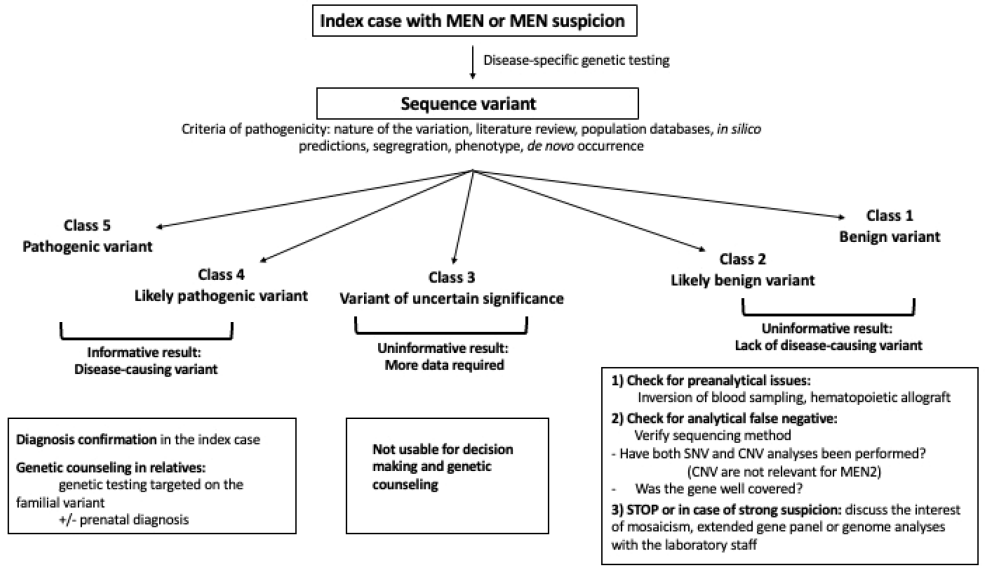

FIGURE 1
Flow chart of genetic testing, interpretation and use of sequence variants in a clinical context. CNV, Copy number variation; SNV, single nucleotide variation.

hand, a VUS should never be used for decision making and genetic counseling. Nevertheless, appropriate follow-up of patients with variants classed as VUS is necessary to check for potential re-classification, which may clearly impact on clinical decision making and subsequent familial genetic counseling.

3.2 MEN1

In MEN1, classical genetic testing on blood DNA is positive in 90% to 95% of familial cases, and in 30-45% of sporadic cases who present with the classical triad (21–23). In patients with 2 MEN1 major lesions, the mutation detection rate is less than 20%, depending on the associated lesions. Nevertheless, the identification of a *MEN1* pathogenic variant is crucial since even though the MEN1 diagnosis can be done on clinical basis, identification of the pathogenic variant is required to inform the genetic counseling, meaning familial presymptomatic genetic testing and in some cases, allowing prenatal diagnosis to be proposed. In MEN1, presymptomatic screening allows a genetic diagnosis to be made, and to then include the MEN1-genetically positive member of the family in a specific follow-up program. For MEN1-genetically negative members, the risk of having MEN1 is then considered equal to the risk in the general population, i.e. a low risk according to the prevalence of the disease, hence they are excluded from specific management. Conceptually, in the absence of identification of *MEN1* pathogenic or likely pathogenic variants in a patient with a clinical or familial diagnosis of MEN1, all of the family members must be considered at risk and be included in the MEN1-specific follow-up program.

MEN1 inactivating mutations can occur throughout the gene sequence. In the UMD-MEN1 database of French patients with *MEN1* variants, microinsertions or deletions have been identified in half of the index cases and missense substitutions in one quarter of cases (24). Splice junction, mid-intronic, and synonymous substitutions are less frequent. Copy number variations (CNV), i.e. large deletions, affecting one or several exons were found in 2.2% of index cases with a *MEN1* variant. The genotype-phenotype relationship remains a matter of debate in MEN1, except for variants affecting the JUND interacting domain (25).

An important question that arises is, since the mutation detection rate is inferior to 20% in index cases with two major lesions of MEN1-2major lesions, do all of the family members need to be included in the MEN1-specific follow-up program if no pathogenic or likely pathogenic variant is identified by genetic testing.

Faced with a genetically-negative MEN1 patient, the first step is to verify that both single nucleotide variations (SNV) and copy number variations (CNV) were searched for during genetic testing (26). CNVs most frequently affect one or more *MEN1* coding exons, however a heterozygous 596bp deletion between nucleotides -1087 and -492 upstream of the translation start site has recently been described, located within the 5' untranslated region (UTR) of *MEN1*, and including the core promoter and multiple cis-regulatory regions (27).

Variations in the deep intronic or promotor regions may also cause MEN1 by altering *MEN1* splicing or expression level. Nevertheless, genome wide analysis can be used to rectify false negative MEN1 genetic testing. For example, Backman et al. sequenced the constitutional genome of fourteen patients with a clinical diagnosis of MEN1 (n = 13) or suspected MEN1 (n = 1) who had negative first-line MEN1 genetic screening (28). They found that three patients carried *MEN1* pathogenic variants (two splice-site variants, one missense variant) that had not been detected during routine clinical sequencing. Analytical false negatives are a side effect of the paradigm change between Sanger sequencing and next generation sequencing (NGS).

Clinical Sanger sequencing requires a manual review of the sequences, which is time-consuming but relatively simple for heterozygous or homozygous variant identification, but requires specific expertise for the detection of unconventional abnormalities such as mosaicism. On the other hand, NGS generates a large amount of data that are mainly treated in an automated manner. Consequently, numerous precautions must be taken using bioinformatics tools to ensure the quality and accuracy of the sequencing. Among these, sequencing depth and coverage are two crucial concepts. Sequencing depth refers to the number of times a specific base in the DNA sequence is read during the sequencing process. For germline analysis, it is generally recommended that a given position is sequenced a minimum of 20 or 30 times to accept that the targeted sequence has been effectively sequenced. This data is assessed by the coverage, the proportion of the target sequence that was sequenced at a certain depth. Less than 100% coverage for a gene of interest may mean that a variant was missed because it was not effectively sequenced. Custom targeted gene panels are most often designed to precisely target the coding sequences and exon-intron junctions of the genes of interest, and thus avoid coverage defects. This is particularly well-suited to the study of specific diseases such as the predisposition to endocrine tumors. It is not always the case with comprehensive hereditary cancer gene panels or whole exome sequencing approaches, which may be chosen by general hospitals to meet the needs of different specialists in a variety of diseases (29).

Variants are extracted by matching the reads, i.e. sequences, against the reference genome, and filtered to avoid background noise and recurrent artifacts. This filtering is based on the mutant allele frequency of the variants, on the coverage, but also on data quality, such as quality value (QV). Indeed, higher sequencing depth provides more confidence in the accuracy of the base calls at that position and helps to reduce sequencing errors and noise. Moreover, during the NGS process, a QV is assigned to each nucleotide in a read. These QVs express the confidence that the corresponding nucleotide call is correct (30). When sequencing quality reaches Q30, virtually all the reads will be perfect, with no errors or ambiguities. This Q30 score is considered a benchmark for quality in NGS, and is frequently used for filtering out potential false positive variants. Unfortunately, some regions of the genome may still be difficult to sequence, such as GC-rich regions that are frequently found at the beginning of genes, including *MEN1* (Figure 2). In these regions, the QV of a true pathogenic variant may be inferior to the threshold and may not pass the filters, leading to a false negative result which can be rectified either by using more permissive bioinformatics analysis or by changing the sequencing technology. Nevertheless, in their genome study, other than the three patients with a pathogenic variant in the coding sequence of *MEN1*, Backman et al. also identified one patient carrying a pathogenic variant in *CASR* and one patient carrying a gross deletion on chromosome 1q which included the *CDC73* gene. Finally, in six patients without mutations, analysis of matched tumor DNA did not detect any recurrent genes fulfilling Knudson's two-hit model. These data showed that deep intronic or promoter pathogenic variants are probably not frequently found in genetically-negative MEN1 patients.

In parallel with the evolution of knowledge about MEN1, other genetic diseases that can give confounding phenotypes, such as MEN4, familial PHPT due to *CDC73* inactivating mutations (31), familial hypocalciuric hypercalcemia type 1, 2, and 3, respectively due to *CASR*, *GNA11*, or *AP2S1* inactivating mutations (32–34), and familial PitNET due to *AIP* (35, 36) inactivating mutations, have been discovered. When faced with patients presenting with isolated MEN1-related lesions at a young age or with atypical clinical presentations, it is necessary to screen not only for variants in *MEN1* but also in other genes that are known to be involved in confounding phenotypes. This has now been made possible thanks to the availability of high-throughput sequencing methods in diagnostic laboratories, even for targeted analysis.

Mosaicism can explain some of the unresolved MEN1 cases (37–39). Mosaicism corresponds to the spontaneous acquisition of a genetic variant during cell division occurring in post-zygotic embryonic development (Figure 3). Mosaicism thus results in a fetus, and then an individual, composed of a variable proportion of cells carrying the mutation, depending on how early and in which cell lines the variant occurs. Mosaic variants may be undetectable in blood samples using classical sequencing methods because the mutated allelic frequency is too low; indeed levels of mosaicism below ~10%–20% are difficult to reliably detect using Sanger sequencing (40).

Only a few cases have been reported to date and the frequency of *MEN1* mosaicism is probably underestimated (38). Indeed, identification of MEN1 mosaicism remains challenging in routine practice in diagnostic laboratories and consequently it is not systematically searched for. Moreover, as *MEN1* mutations can occur along the entire length of the gene, it is not possible to use a sensitive targeted method such as digital droplet PCR in first line. NGS offers new possibilities for detecting mosaic variants (41). However, low-frequency variants are often filtered out as they are difficult to distinguish from background noise in bioinformatics pipelines. In a previous study, we evaluated the performance of NGS with unique molecular identifiers (UMI) in the diagnosis of *MEN1* mosaicism in routine practice. UMIs are unique oligonucleotide sequences which are added to DNA prior to any amplification and they differentially label each molecule in the native DNA fragment. UMIs allow for a computational correction of amplification bias and sequencing errors that improves molecular detection of rare events. Among a cohort of 119 patients harboring from 2 to 5 MEN1-related lesions, we identified 3 patients with *MEN1* mosaic pathogenic variants. The mutated allele frequencies ranged from 2.3 to 9.5%. The detection rate of *MEN1* mosaicism in patients bearing at least 3 MEN1 lesions was 17% (3/18). No cases were detected in patients with 2 lesions.

The last aspect affecting accurate MEN1 molecular diagnosis is the assessment of the pathogenicity of *MEN1* variants. In the last ten years, significant efforts have been made to standardize and harmonize the criterion for classification of sequence variants (42). The main benefit is an improvement in the robustness of classification, from expert opinion to evidence-based medicine. For many years, the Human Genome Variation Society and others scientific groups have promoted the use of the 5 pathogenicity classes to describe variants identified in genes that cause Mendelian

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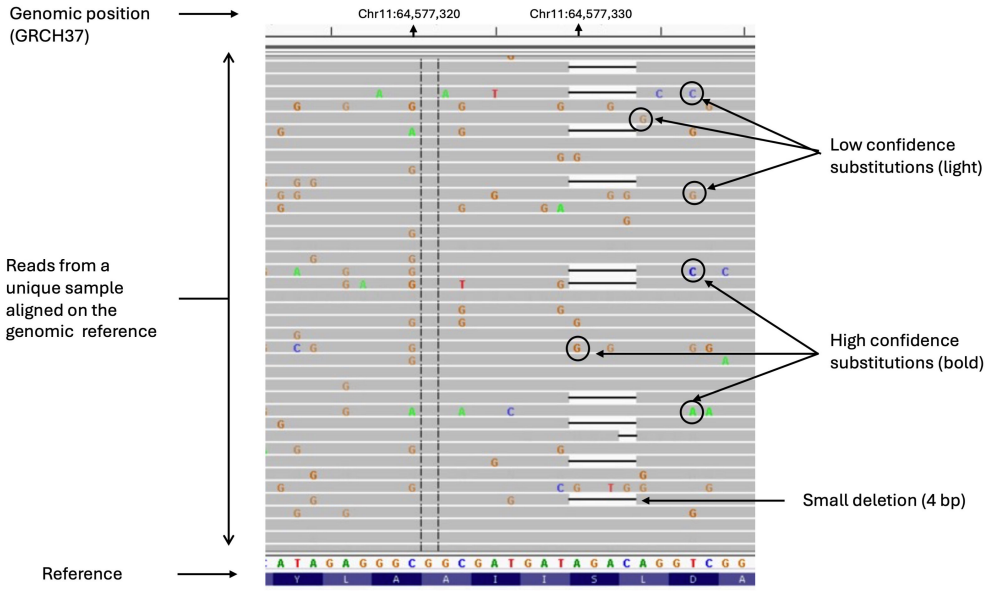

FIGURE 2
Read alignments produced on an Illumina MiSeq sequencer and visualized using IGV 2.11.3. The reads show a GC-rich region in the second exon of the *MEN1* gene. The top bar indicates chromosomal coordinates. Bold letters indicate mismatch bases corresponding to putative variants. Color intensity indicates the sequencing quality values. Clear letters denote poor sequencing quality scores. In these regions, the quality of sequencing is suboptimal leading to potential false-positive results. To avoid this, variants are filtered out based on the quality value, which can also lead to rejection of a true variant if its quality is insufficient. Here the deletion c.249_252del heterozygous of four bases (indicated by the horizontal black line) was filtered out in first-line analysis because the quality value attributed to an absence of signal (deletion) is the quality value of the surrounding region.

disorders. This classification reflects the diversity of the human genome and the state of scientific and medical knowledge. In 2015, the ACMG-AMP Variant-Interpretation Guidelines revolutionized the evaluation of sequence variants in human Mendelian diseases (42). These guidelines provide a general procedure for an evidence framework including 28 classification criteria divided into 8 evidence types: "population data", "computational and predictive data", "functional data", "segregation data", "*de novo* data", "allelic data", "other database" or "other data" (including clinical data). Each criterion is weighted for a benign effect (stand-alone evidence of benign impact: BA; strong evidence of benign impact: BS, supporting evidence of benign impact: BP) or a pathogenic effect (very strong evidence of pathogenicity: PVS; strong evidence of pathogenicity: PS; moderate evidence of pathogenicity: PM; supporting evidence of

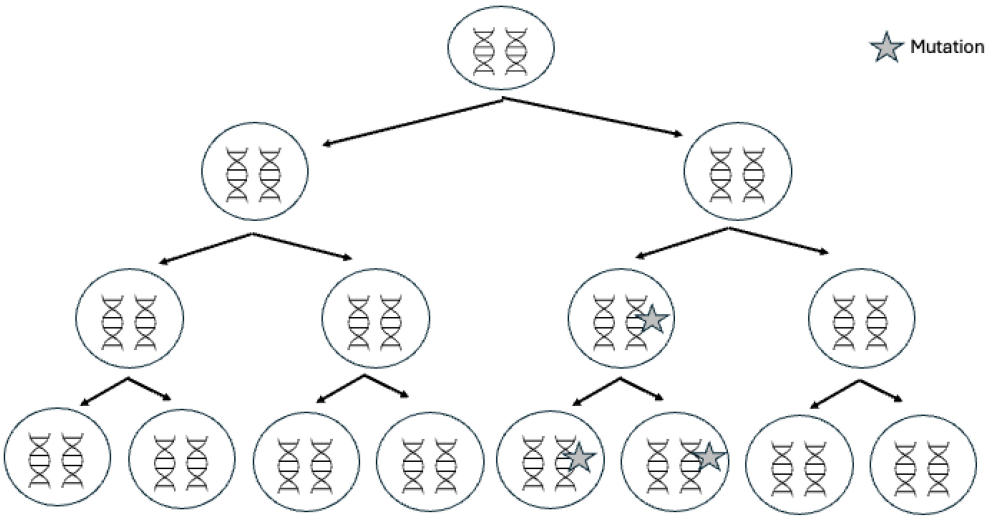

FIGURE 3
Genetic mosaicism. Mosaicism is due to the occurrence of a *de novo* mutation during postzygotic development, after fertilization. In these cases, only a proportion of the cells will harbor the variation; this feature is termed mosaicism or somatic mosaicism.

pathogenicity: PP), for examples: criterion PM1 (moderate evidence of pathogenicity): variant located in a mutational hot spot and/or critical and well-established functional domain (e.g., active site of an enzyme) without benign variation, criterion BS3 (strong evidence of benign impact): variant with well-established *in vitro* or *in vivo* functional studies showing no deleterious effect on protein function or splicing. The compilation of evidence scores led to more objective and reproducible classification of variations into one of the five classification classes (examples: 1 PVS criterion + 1 PS criterion or 1 PS criterion + 3 PM criteria lead to a PV classification, 2 PM+ 2 PP: LPV classification, 2BS: LBV). When the criteria for benign or pathogenic variant are insufficient or contradictory, the variant is classified as a variant of uncertain significance.

We have observed that the ACMG-AMP classification leads to an over-classification of variants as VUS compared to the classification by a consortium of experts, especially for *MEN1* missense variants, which is the reason we have proposed adjustment to this classification for *MEN1* missense variants (43).

On the other hand, the ACMG-AMP has also made it possible to reclassify variants, wrongly considered as pathogenic, into benign or likely benign variants. For example, the variant NM_130799.2 (*MEN1*):c.1618C>T, p.(Pro540Ser) was subsequently classified as pathogenic, likely pathogenic, and variant of uncertain significance in different studies (44–46). A comprehensive study, including general population data, family co-segregation study, and tumor loss of heterozygosity analysis finally led to this variant being classified as benign (47). For patients, if MEN1 is still suspected based on clinical data, even in the case of a first-round negative genetic result, genetic analysis should be continued, for example by searching for CNV or mosaicism.

3.3 MEN2

There are few pitfalls in the diagnosis of MEN2 since MEN2 results from well-known recurrent punctual variations. The identification of a *RET* mutation at the somatic or germline level is essential since RET inhibitors are now used in the management of MTCs. If a MEN2-related pathogenic variant is identified in a MTC at the somatic level, it is important to check at the germline level to rule out MEN2. Indeed, MTC is due to MEN2A in 16% of cases (48).

3.4 MEN4

CDKN1B variants are less frequently identified in MEN1-suspected patients than *MEN1* variants (16). Nevertheless, the variant interpretation is subject to the same issues, especially the low and late penetrance of the disease making familial segregation studies difficult to interpret. On the other hand, several teams have developed *in vitro* analyses based on the nuclear localization of the $P27^{KIP1}$ protein, encoded by *CDKN1B*, the degradation kinetics of the protein, or based on the molecular function of this protein (49–53). Unconventional variants may also be identified in MEN4 patients. Indeed, in 2013 Occhi and al identified a variant located in the 5' untranslated region (5'UTR) of the *CDKN1B* mRNA, in a highly conserved regulatory upstream open reading frame (uORF). This variant, creates a frameshift and elongation of this uORF, leading to reduced $P27^{KIP1}$ activity.

4 New MENs

The association between PA and PPGL was first described by Iversen in 1952, and can occur in clinical MEN1 or independently (54). The condition, termed the "3PAs" syndrome (for PitNET/pheochromocytoma/paraganglioma association) by Xekouki, can be described as the co-occurrence of PA and PPGL without other features of MEN1 syndrome (55). This association is rare, with fewer than 200 cases published to date. In half of the cases, an association with a genetic variant has been reported, mainly in genes involved in predisposition to pheochromocytoma and paraganglioma such as *SDHA* (OMIM 614165), *SDHB* (OMIM 115310), *SDHC* (OMIM 605373) and *SDHD* (OMIM 168000) genes coding for the subunits of succinate dehydrogenase, and *MAX* (OMIM 171300) (46, 55–59). Though the association between *SDHx* variants and PitNETs seems to be proven thanks to evidence from animal models, tumor analysis, and *in vivo* analysis such as 1H-RM spectroscopy (56, 60), pituitary tumorigenesis in patients with an *SDHx* pathogenic variant does not seem to be systematically due to a somatic second hit according to the Knudson model (61). Moreover, the frequency of putative *SDHx* pathogenic variants in the general population, assessed by the frequency of loss of function variants in genome database like gnomAD, compared to the rarity of this condition questions the benefit of PitNET screening in carriers of *SDHx* mutations.

On the other hand, several patients have been reported with a *MAX* pathogenic or likely pathogenic variant and a NET other than pheochromocytoma in the literature. *MAX* codes for the MAX protein, a component of the MYC signaling pathway. The protein forms heterodimers with C-MYC via basic-helix-loop-helix zipper domain interactions. These heterodimers can then bind to target DNA sequences or E-BOX sequences to regulate transcription of genes involved in cell proliferation and cell growth. *MAX* behaves as a tumor suppressor gene. Germline and somatic *MAX* variants can result in PPGL (62). To date, at least seven patients with *MAX* variants and PitNET have been reported, six of whom had functional PitNETs (4 lactotroph PitNETs, 2 somatotroph PitNETs) (59, 63, 64).

Other NETs have been reported in patients carrying *MAX* mutations with or without PitNETs, including ganglioneuroma, ganglioneuroblastoma, adrenomedullary hyperplasia, pancreatic NETs, and parathyroid adenomas, but also nonendocrine tumors, including renal oncocytomas, renal carcinomas, breast carcinomas, and squamous cell tumors (62, 64–67). Nevertheless, the causal link between these non-PPGL tumors and *MAX* variants remains to be established.

The *CDC73* gene is also suspected to be implicated in MEN1 phenocopy, since the report of a patient with a clinical diagnosis of MEN1, based on the combined occurrence of normocalcemic PHPT at age 70 years, acromegaly diagnosed at age 22 years, and

a pancreatic NET at age 70 years, harboring a heterozygous c.1138C>T p.(Leu380Phe) *CDC73* germline variant suspected to be pathogenic (68). Characterization of the pancreatic tumor confirmed the neuroendocrine origin of the neoplasm with positive immunostaining for chromogranin and glucagon. *CDC73* is a tumor suppressor gene. *CDC73* germline pathogenic variants are responsible for hyperparathyroidism-jaw tumor (HPT-JT) syndrome (OMIM 145001), which associates parathyroid adenoma or carcinoma, fibro-osseous jaw tumor, cystic kidney lesion, uterine tumors and in rare cases tumors of the thyroid, testis, and pituitary in a single patient (31, 69, 70). In a report by Lines et al. (68), the p.(Leu380Phe)*CDC73* variant was suspected of pathogenicity because the variant (i) occurred in a highly conserved residue, (ii) is involved in the interaction domain, (iii) is absent from 120,000 individuals in the gnomAD database, and (iv) is predicted to have a damaging effect by computational analysis. Concerning the involvement of this *CDC73* variant in pancreatic NETs, an RNA-Scope analysis showed a significant reduction in *CDC73* expression (13.5% ($p<0.005$)), compared to the peritumoral normal pancreas. However, this decreased expression was not shown by immunohistochemistry and questions the link between *CDC73* and the pancreatic tumor in this patient.

5 Gene-disease relationship assessment in the era of genomic medicine

Advances in genomic sequencing technology have led to the number of new gene-disease relationships rapidly expanding. However, the evidence supporting these claims varies widely, often without an accurate evaluation of genomic variations in a clinical setting. The NIH-funded Clinical Genome Resource (ClinGen) has developed a framework to define and evaluate the clinical validity of gene-disease pairs across a variety of Mendelian disorders (71). They first defined six classes to qualitatively describe the strength of evidence supporting a gene-disease association. For example, the evidence to support a causal role for a gene in a disease is considered as limited, (i) if there are fewer than three observations of variants that provide convincing evidence for disease causality, or (ii) if variants have been observed in probands, but none have sufficient evidence for disease causality, and (iii) if there is limited experimental data supporting the gene-disease association. In contrast, variants that disrupt function (such as truncating variants in tumor suppressor genes) and/or that are associated with other strong evidence in genetics or in population data (e.g. *de novo* occurrence, absence in large control cohorts such as the gnomAD database, strong linkage to a small genomic interval, etc.) are considered convincing evidence of disease causality. The authors developed a semiquantitative approach to evaluate both genetic and experimental evidence in a standardized manner that promotes consistent collection and weighting of evidence. Genetic evidence is evaluated based on case information: (i) *de novo* occurrence of the suspected variant, (ii) variants causing loss of function, (iii) evidence of segregation in one or more families, and (iv) case-control study data, provided that quality criteria are met, such as a sufficient number of cases and controls given the prevalence of the disease, case-control matching limiting bias and confounding factors (the same demographic information between cases and controls, the same phenotypical evaluation…), taking into account multiple testing for statistical significance, the use of methods for variant detection with equivalent analytical performance in cases and controls. For example, we can assign a stronger level of confidence for the pathogenicity of a *MAX* variant in a patient with multiple endocrine neoplasia if other candidate genes were ruled out, especially if genome-wide analysis was performed. Conversely, the finding of another pathogenic variant in a gene with a well-established gene-disease association decreases the confidence level. In 2021, Raygada et al. reported the case of a woman with an adrenocortical carcinoma associated with double germline mutations in *MSH2* and *RET* (72). *MSH2* is one of the family of DNA mismatch repair genes, a group of tumor suppressor genes that are involved in Lynch syndrome, a syndrome that predisposes subjects to colorectal and endometrial cancers, as well as adrenocortical carcinoma. The patient carried the *RET* variant c.2410G>A, p.Val804Met is a well-known activating mutation involved in MEN2A with a moderate risk of MTC and an incidence of pheochromocytoma and primary hyperparathyroidism of less than 10%. She also carried a deleterious germline mutation in the *MSH2* gene c.211+1G>T, p.(?) affecting splicing, that was found with a loss of heterozygosity in the adrenal tumor, ruling out the potential involvement of the *RET* mutant in this tumor, and thus representing only an incidental finding.

Experimental evidence also needs also to be evaluated and ranked. Obviously, it is important that the function of the candidate gene product is consistent with the phenotype of an affected individual or has a similar function to another gene known to be involved in the same disease. For tumor suppressor genes, examining for the loss of protein expression by immunohistochemistry and/or the occurrence of a second hit in the tumor should be performed, as a minimum, to document the alteration of gene function. Nevertheless, this criterion is not sufficient to provide either a strong level of evidence or to definitively rule out a gene, because interpretation of the results may be complicated by potential pitfalls including an inappropriate target for immunostaining, a dose effect of protein expression, or because the second hit could be missed if it is an epigenetic event that cannot be detected by conventional sequencing. On the other hand, loss of heterozygosity can be due to large chromosome remodeling that fortuitously includes the candidate gene. For this reason, ideally, we should assess the alteration of function of the protein in human genetically-modified cells, and in animal or non-human cell-culture models with a similarly disrupted copy of the affected gene. The aim of these experiments is to observe a phenotype in these models that is consistent with the human disease state, and potentially rescuing the phenotype in cells derived from affected individuals or engineered equivalents through the addition of the wild-type gene product or correction by gene editing.

Over the past two decades, preclinical research has turned increasingly more to cultured spheroids, tumoroids and organoids to investigate tissue pathophysiology and responses to current and novel drugs therapies (73, 74). Organoids are heterogeneous self-organizing 3D aggregates that can recapitulate the structure,

function, and thereby overall biological complexity of organs, mainly obtained after redifferentiation of induced pluripotent stem cells (iPSC) (75). Tumoroids are generated from patient tumor samples where the various cell types can aggregate *in vitro* and recreate the tumor microenvironment. Spheroids are similar, but typically arise from the aggregation of one cell type, such as immortalized cell lines. In 2022, Noltes et al. generated a patient-derived parathyroid organoid model from hyperplastic parathyroid gland biopsies and showed that the parathyroid organoid model recapitulated the tissue at the gene and protein levels and showed appropriate responses to different calcium concentrations and drugs (76). In 2023, Mallick et al. used genetically-engineered iPSC derived organoids to model the development of corticotroph PitNETs expressing *USP48* or *USP8* somatic mutations (73). Several groups have generated MEN1 –patient-derived iPSC, showing that it is possible to reprogram cells from patients with rare endocrine diseases (77, 78). These technologies could one day be used to test new candidate genes.

In parallel, large cohort studies must be undertaken to better understand the natural history, expressivity, and penetrance of rare endocrine diseases. Indeed, though MEN1 and MEN2 are well-characterized diseases (12, 14), there are relatively few large series describing the phenotype of patients carrying *CDC73*- or *MAX* mutations with follow-up data. Such epidemiological data would enable precise phenotypic characterization, including the assessment of tumor risk according to gene, age or variant, and finally would allow patients to be included in personalized precision medicine programs as well as allowing accurate genetic counseling to be proposed for families.

6 Conclusion

With advances in genomic sequencing technology, the number of reported variants and gene-disease relationships has rapidly expanded. Since not all sequencing technologies are universally available and individual laboratories may choose different strategies, physicians must be trained to understand the aims, limits, advantages, and pitfalls of genetic testing, so that patients with these rare diseases do not risk an incorrect diagnosis due to the failure to perform further genetic analyses. On the other hand, efforts must be made at the regional and national levels to establish molecular diagnostics networks to optimize genetic diagnosis in difficult cases. Variants in disease causing genes must be carefully and regularly evaluated according to the current state of the art data. In patients carrying known pathogenic variants, the occurrence of tumors outside the known tumor gene spectrum could reveal a new gene-tumor association or may be due to another genetic origin, including another genetic disease. In any case, experimental and epidemiological studies must be conducted into rare endocrine diseases to better characterize the links between known and new candidate genes and multiple endocrine and non-endocrine neoplasia in these patients.

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References

1. Wermer P. Genetic aspects of adenomatosis of endocrine glands. *Am J Med.* (1954) 16:363–71. doi: 10.1016/0002-9343(54)90353-8

2. Evans JP, Skrzynia C, Burke W. The complexities of predictive genetic testing. *BMJ (Clinical Res ed.).* (2001) 322:1052–6. doi: 10.1136/BMJ.322.7293.1052

3. Pellegata NS, Quintanilla-Martinez L, Siggelkow H, Samson E, Bink K, Höfler H, et al. Germ-line mutations in p27Kip1 cause a multiple endocrine neoplasia syndrome in rats and humans. *Proc Natl Acad Sci United States America.* (2006) 103:15558–63. doi: 10.1073/PNAS.0603877103

4. Brandi ML, Agarwal SK, Perrier ND, Lines KE, Valk GD, Thakker RV. Multiple endocrine neoplasia type 1: latest insights. *Endocr Rev.* (2021) 42:133–70. doi: 10.1210/endrev/bnaa031

5. Wang P, Lin C, Smith ER, Guo H, Sanderson BW, Wu M, et al. Global analysis of H3K4 methylation defines MLL family member targets and points to a role for MLL1-mediated H3K4 methylation in the regulation of transcriptional initiation by RNA polymerase II. *Mol Cell Biol.* (2009) 29:6074–85. doi: 10.1128/MCB.00924-09

6. Huang J, Gurung B, Wan B, Matkar S, Veniaminova NA, Wan K, et al. The same pocket in menin binds both MLL and JUND but has opposite effects on transcription. *Nature.* (2012) 482:542–6. doi: 10.1038/NATURE10806

7. Canaff L, Vanbellinghen JF, Kaji H, Goltzman D, Hendy GN. Impaired transforming growth factor-β (TGF-β) transcriptional activity and cell proliferation control of a menin in-frame deletion mutant associated with multiple endocrine neoplasia type 1 (MEN1). *J Biol Chem.* (2012) 287:8584–97. doi: 10.1074/jbc.M112.341958

8. Agarwal SK, Guru SC, Heppner C, Erdos MR, Collins RM, Park SY, et al. Menin interacts with the AP1 transcription factor JunD and represses JunD-activated transcription. *Cell.* (1999) 96:143–52. doi: 10.1016/S0092-8674(00)80967-8

9. Knudson AG Jr. Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci United States A*. (1971) 68:820–3. doi: 10.1073/pnas.68.4.820

10. Goudet P, Murat A, Binquet C, Cardot-Bauters C, Costa A, Ruszniewski P, et al. Risk Factors and Causes of Death in MEN1 Disease. A GTE (Groupe d'Etude des Tumeurs Endocrines) Cohort Study Among 758 Patients. *World J Surg*. (2010) 34:249–55. doi: 10.1007/s00268-009-0290-1

11. Dean PG, van Heerden JA, Farley DR, Thompson GB, Grant CS, Harmsen WS, et al. Are patients with multiple endocrine neoplasia type I prone to premature death? *World J Surg*. (2000) 24:1437–41. doi: 10.1007/s002680010237

12. Thakker RV, Newey PJ, Walls GV, Bilezikian J, Dralle H, Ebeling PR, et al. Clinical practice guidelines for multiple endocrine neoplasia type 1 (MEN1). *J Clin Endocrinol Metab*. (2012) 97:2990–3011. doi: 10.1210/jc.2012-1230

13. van Treijen MJC, de Vries LH, Hertog D, Vriens MR, Stuart AAV. Nesselrooij BPM van, & Valk GD. Multiple endocrine neoplasia type 2. *Endotext*. (2022).

14. Wells SA, Asa SL, Dralle H, Elisei R, Evans DB, Gagel RF, et al. Revised American thyroid association guidelines for the management of medullary thyroid carcinoma. *Thyroid*. (2015) 25:567–610. doi: 10.1089/thy.2014.0335

15. Morrison PJ, Nevin NC. Multiple endocrine neoplasia type 2B (mucosal neuroma syndrome, Wagenmann-Froboese syndrome). *J Med Genet*. (1996) 33:779–82. doi: 10.1136/JMG.33.9.779

16. Chevalier B, Coppin L, Romanet P, Cuny T, Maïza JC, Abeillon J, et al. Correction to: "Beyond MEN1, when to think about MEN4? Retrospective study on 5600 patients in the French population & literature review". *J Clin Endocrinol Metab*. (2024) 109:e1482–e1493. doi: 10.1210/CLINEM/DGAE055

17. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci United States A*. (1977) 74:5463–7. doi: 10.1073/PNAS.74.12.5463

18. Korf BR, Rehm HL. New approaches to molecular diagnosis. *JAMA*. (2013) 309:1511–21. doi: 10.1001/JAMA.2013.3239

19. Den Dunnen JT, Dalgleish R, Maglott DR, Hart RK, Greenblatt MS, Mcgowan-Jordan J, et al. HGVS recommendations for the description of sequence variants: 2016 update. (2016) 37(6):564–9. doi: 10.1002/humu.22981

20. Joynt ACM, Axford MM, Chad L, Costain G. Understanding genetic variants of uncertain significance. *Paediatr Child Health*. (2022) 27:10. doi: 10.1093/PCH/PXAB070

21. Lemos MC, Thakker RV. Multiple endocrine neoplasia type 1 (MEN1): analysis of 1336 mutations reported in the first decade following identification of the gene. *Hum Mutation*. (2008) 29:22–32. doi: 10.1002/humu.20605

22. Odou MF, Cardot-Bauters C, Vantyghem MC, Carnaille B, Leteurtre E, Pigny P, et al. Contribution of genetic analysis in screening for MEN1 among patients with sporadic disease and one or more typical manifestation. *Annales D'endocrinol*. (2006) 67:581–7. doi: 10.1016/S0003-4266(06)73010-4

23. Tham E, Grandell U, Lindgren E, Toss G, Skogseid B, Nordenskjöld M. Clinical testing for mutations in the MEN1 gene in Sweden: a report on 200 unrelated cases. *J Clin Endocrinol Metab*. (2007) 92:3389–95. doi: 10.1210/jc.2007-0476

24. Romanet P, Mohamed A, Giraud S, Odou MF, North MO, Pertuit M, et al. UMD-MEN1 database: an overview of the 370 *MEN1* variants present in 1676 patients from the French population. *J Clin Endocrinol Metab*. (2019) 104:753–64. doi: 10.1210/jc.2018-01170

25. Thevenon J, Bourredjem A, Faivre L, Cardot-Bauters C, Calender A, Murat A, et al. Higher risk of death among MEN1 patients with mutations in the JunD interacting domain: a Groupe d'etude des Tumeurs Endocrines (GTE) cohort study. *Hum Mol Genet*. (2013) 22:1940–8. doi: 10.1093/hmg/ddt039

26. Ellard S, Hattersley AT, Brewer CM, Vaidya B. Detection of an MEN1 gene mutation depends on clinical features and supports current referral criteria for diagnostic molecular genetic testing. *Clin Endocrinol*. (2005) 62:169–75. doi: 10.1111/j.1365-2265.2005.02190.x

27. Kooblall KG, Boon H, Cranston T, Stevenson M, Pagnamenta AT, Rogers A, et al. Multiple endocrine neoplasia type 1 (MEN1) 5'UTR deletion, in MEN1 family, decreases menin expression. *J Bone Mineral Res: Off J Am Soc Bone Mineral Res*. (2021) 36:100–9. doi: 10.1002/JBMR.4156

28. Backman S, Bajic D, Crona J, Hellman P, Skogseid B, Stålberg P. Whole genome sequencing of apparently mutation-negative MEN1 patients. *Eur J Endocrinol*. (2020) 182:35–45. doi: 10.1530/EJE-19-0522

29. Aisagbonhi O, Ghlichloo I, Hong DS, Roma A, Fadare O, Eskander R, et al. Comprehensive next-generation sequencing identifies novel putative pathogenic or likely pathogenic germline variants in patients with concurrent tubo-ovarian and endometrial serous and endometrioid carcinomas or precursors. *Gynecol Oncol*. (2024) 187:241–8. doi: 10.1016/j.ygyno.2024.05.027

30. Ewing B, Hillier LD, Wendl MC , Green P. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res*. (1998) 8:175–85. doi: 10.1101/gr.8.3.175

31. Newey PJ, Bowl MR, Cranston T, Thakker RV. Cell division cycle protein 73 homolog (CDC73) mutations in the hyperparathyroidism-jaw tumor syndrome (HPT-JT) and parathyroid tumors. *Hum Mutation*. (2010) 31:295–307. doi: 10.1002/humu.21188

32. Hannan FM, Nesbit MA, Zhang C, Cranston T, Curley AJ, Harding B, et al. Identification of 70 calcium-sensing receptor mutations in hyper- and hypo-calcaemic patients: Evidence for clustering of extracellular domain mutations at calcium-binding sites. *Hum Mol Genet*. (2012) 21:2768–78. doi: 10.1093/hmg/dds105

33. Nesbit MA, Hannan FM, Howles SA, Reed AAC, Cranston T, Thakker CE, et al. Mutations in AP2S1 cause familial hypocalciuric hypercalcemia type 3. *Nat Genet*. (2013) 45:93–7. doi: 10.1038/NG.2492

34. Andrew Nesbit M, Hannan FM, Howles SA, Babinsky VN, Head RA, Cranston T, et al. Mutations affecting G-protein subunit α11 in hypercalcemia and hypocalcemia. *New Engl J Med*. (2013) 368:2476–86. doi: 10.1056/NEJMOA1300253

35. Vierimaa O, Georgitsi M, Lehtonen R, Vahteristo P, Kokko A, Raitila A, et al. Pituitary adenoma predisposition caused by germline mutations in the AIP gene. *Sci (New York NY)*. (2006) 312:1228–30. doi: 10.1126/science.1126100

36. Daly AF, Vanbellinghen JF, Sok KK, Jaffrain-Rea ML, Naves LA, Guitelman MA, et al. Aryl hydrocarbon receptor-interacting protein gene mutations in familial isolated pituitary adenomas: analysis in 73 families. *J Clin Endocrinol Metab*. (2007) 92:1891–6. doi: 10.1210/JC.2006-2513

37. Coppin L, Giraud S, Pasmant E, Lagarde A, North MO, Le-Collen L, et al. Multiple endocrine neoplasia type 1 caused by mosaic mutation: clinical follow-up and genetic counseling? *Eur J Endocrinol*. (2022) 187:K1–6. doi: 10.1530/EJE-22-0171

38. Lagarde A, Mougel G, Coppins L, Haissaguerre M, Le Collen L, Mohamed A, et al. Systematic detection of mosaicism by using digital NGS reveals three new MEN1 mosaicisms. *Endocr Connections*. (2022) 11(11):e220093. doi: 10.1530/EC-22-0093

39. Lagarde A, Le Collen L, Boulagnon C, Brixi H, Durlach A, Mougel G, et al. Early detection of relapse by ctDNA sequencing in a patient with metastatic thymic tumor and MEN1 mosaicism. *J Clin Endocrinol Metab*. (2022) 107:e4154–8. doi: 10.1210/clinem/dgac454

40. Mefford HC. Mosaicism in clinical genetics. *Cold Spring Harbor Mol Case Stud*. (2021) 7:a006162. doi: 10.1101/MCS.A006162

41. Coppin L, Grutzmacher C, Crépin M, Destailleur E, Giraud S, Cardot-Bauters C, et al. VHL mosaicism can be detected by clinical next-generation sequencing and is not restricted to patients with a mild phenotype. *Eur J Hum Genet*. (2014) 22:1149–52. doi: 10.1038/ejhg.2013.279

42. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. (2015) 17:405–24. doi: 10.1038/gim.2015.30

43. Romanet P, Odou M, North M, Saveanu A, Coppin L, Pasmant E, et al. Proposition of adjustments to the ACMG-AMP framework for the interpretation of *MEN1* missense variants. *Hum Mutation*. (2019) 40:661–74. doi: 10.1002/humu.23746

44. Crépin M, Escande F, Pigny P, Buisine MP, Calender A, Porchet N, et al. Efficient mutation detection in MEN1 gene using a combination of single-strand conformation polymorphism (MDGA) and heteroduplex analysis. *Electrophoresis*. (2003) 24:26–33. doi: 10.1002/elps.200390023

45. Cuny T, Pertuit M, Sahnoun-Fathallah M, Daly A, Occhi G, Odou MF, et al. Genetic analysis in young patients with sporadic pituitary macroadenomas: besides AIP don't forget MEN1 genetic analysis. *Eur J Endocrinol*. (2013) 168:533–41. doi: 10.1530/EJE-12-0763

46. Guerrero-Pérez F, Fajardo C, Torres Vela E, Giménez-Palop O, Lisbona Gil A, Martín T, et al. 3P association (3PAs): Pituitary adenoma and pheochromocytoma/paraganglioma. A heterogeneous clinical syndrome associated with different gene mutations. *Eur J Internal Med*. (2019) 69:14–9. doi: 10.1016/j.ejim.2019.08.005

47. Villabona C, Oriola J, Serrano T, Guerrero-Pérez F, Valdés N, Chiara M, et al. The recurrent p.(Pro540Ser) MEN1 genetic variant should be considered nonpathogenic: A case report. *Am J Med Genet Part A*. (2021) 185:3872–6. doi: 10.1002/AJMG.A.62444

48. Xu B, Viswanathan K, Ahadi MS, Ahmadi S, Alzumaili B, Bani MA, et al. Association of the genomic profile of medullary thyroid carcinoma with tumor characteristics and clinical outcomes in an international multicenter study. *Thyroid: Off J Am Thyroid Assoc*. (2024) 34:167–76. doi: 10.1089/THY.2023.0279

49. Tichomirowa MA, Lee M, Barlier A, Daly AF, Marinoni I, Naves LA, et al. Cyclin-dependent kinase inhibitor 1B (CDKN1B) gene variants in AIP mutation-negative familial isolated pituitary adenoma kindreds. *Endocr Relat Cancer*. (2012) 19 (3):233–41. doi: 10.1530/ERC-11-0362

50. Chasseloup F, Pankratz N, Lane J, Faucz FR, Keil MF, Chittiboina P, et al. Loss-of-function variants cause pediatric Cushing's disease with or without an MEN4 phenotype. *J Clin Endocrinol Metab*. (2020) 105(6):1983–2005. doi: 10.1210/clinem/dgaa160

51. Malanga D, De Gisi S, Riccardi M, Scrima M, De Marco C, Robledo M, et al. Functional characterization of a rare germline mutation in the gene encoding the cyclin-dependent kinase inhibitor p27Kip1 (CDKN1B) in a Spanish patient with multiple endocrine neoplasia-like phenotype. *Eur J Endocrinol*. (2012) 166(3):551–60. doi: 10.1530/EJE-11-0929

52. Pardi E, Mariotti S, Pellegata NS, Benfini K, Borsari S, Saponaro F, et al. Functional characterization of a CDKN1B mutation in a Sardinian kindred with multiple endocrine neoplasia type 4. *Endocr Connect*. (2015) 4(1):1–8. doi: 10.1530/EC-14-0116

53. Molatore S, Marinoni I, Lee M, Pulz E, Ambrosio MR, degl Uberti EC, et al. A novel germline CDKN1B mutation causing multiple endocrine tumors: clinical, genetic and functional characterization. *Hum Mutat*. (2010) 31(11):E1825-35. doi: 10.1002/HUMU.21354

54. IVERSEN K. Acromegaly associated with phaeochromocytoma. *Acta Med Scand*. (1952) 142:1–5. doi: 10.1111/J.0954-6820.1952.TB13837.X

55. Xekouki P, Pacak K, Almeida M, Wassif CA, Rustin P, Nesterova M, et al. Succinate dehydrogenase (SDH) D subunit (SDHD) inactivation in a growth-hormone-producing pituitary tumor: a new association for SDH? *J Clin Endocrinol Metab*. (2012) 97:E357–66. doi: 10.1210/jc.2011-1179

56. Xekouki P, Szarek E, Bullova P, Giubellino A, Quezado M, Mastroyannis SA, et al. Pituitary adenoma with paraganglioma/pheochromocytoma (3PAs) and succinate dehydrogenase defects in humans and mice. *J Clin Endocrinol Metab*. (2015) 100:E710–9. doi: 10.1210/jc.2014-4297

57. Dénes J, Swords F, Rattenberry E, Stals K, Owens M, Cranston T, et al. Heterogeneous genetic background of the association of pheochromocytoma/paraganglioma and pituitary adenoma: results from a large patient cohort. *J Clin Endocrinol Metab* 100(3):E531-41. doi: 10.1210/jc.2014-3399

58. O'Toole SM, Dénes J, Robledo M, Stratakis CA, Korbonits M. The association of pituitary adenomas and phaeochromocytomas or paragangliomas. *Endocr Rel Cancer*. (2015) 22:T105–22. doi: 10.1530/ERC-15-0241

59. Daly AF, Castermans E, Oudijk L, Guitelman MA, Beckers P, Potorac I, et al. Pheochromocytomas and pituitary adenomas in three patients with MAX exon deletions. *Endocr Rel Cancer*. (2018) 25:L37–42. doi: 10.1530/ERC-18-0065

60. Branzoli F, Salgues B, Marjańska M, Laloi-Michelin M, Herman P, Le Collen L, et al. SDHx mutation and pituitary adenoma: can in *vivo* 1H-MR spectroscopy unravel the link? *Endocr Rel Cancer*. (2023) 30(2):e220198. doi: 10.1530/ERC-22-0198

61. Mougel G, Lagarde A, Albarel F, Essamet W, Luigi P, Mouly C, et al. Germinal defects of SDHx genes in patients with isolated pituitary adenoma. *Eur J Endocrinol*. (2020) 183:369–79. doi: 10.1530/EJE-20-0054

62. Burnichon N, Cascón A, Schiavi F, Morales NP, Comino-Méndez I, Abermil N, et al. MAX mutations cause hereditary and sporadic pheochromocytoma and paraganglioma. *Clin Cancer Res: an Off J Am Assoc Cancer Res*. (2012) 18:2828–37. doi: 10.1158/1078-0432.CCR-12-0160

63. Roszko KL, Blouch E, Blake M, Powers JF, Tischler AS, Hodin R, et al. Case report of a prolactinoma in a patient with a novel MAX mutation and bilateral pheochromocytomas. *J Endocr Society*. (2017) 1:1401–7. doi: 10.1210/JS.2017-00135

64. Seabrook AJ, Harris JE, Velosa SB, Kim E, McInerney-Leo AM, Dwight T, et al. Multiple endocrine tumors associated with germline MAX mutations: multiple endocrine neoplasia type 5? *J Clin Endocrinol Metab*. (2021) 106:e1163–82. doi: 10.1210/CLINEM/DGAA957

65. Romanet P, Guerin C, Pedini P, Essamet W, Castinetti F, Sebag F, et al. Pathological and genetic characterization of bilateral adrenomedullary hyperplasia in a patient with germline MAX mutation. *Endocr Pathol*. (2017) 28:302–7. doi: 10.1007/s12022-016-9460-5

66. Pozza C, Sesti F, Di Dato C, Sbardella E, Pofi R, Schiavi F, et al. A novel MAX gene mutation variant in a patient with multiple and "Composite" Neuroendocrine–neuroblastic tumors. *Front Endocrinol*. (2020) 11:234/PDF. doi: 10.3389/FENDO.2020.00234/PDF

67. Korpershoek E, Koffy D, Eussen BH, Oudijk L, Papathomas TG, Van Nederveen FH, et al. Complex MAX rearrangement in a family with Malignant pheochromocytoma, renal oncocytoma, and erythrocytosis. *J Clin Endocrinol Metab*. (2016) 101:453–60. doi: 10.1210/JC.2015-2592

68. Lines KE, Nachtigall LB, Dichtel LE, Cranston T, Boon H, Zhang X, et al. Multiple endocrine neoplasia type 1 (MEN1) phenocopy due to a cell cycle division 73 (CDC73) variant. *J Endocr Society*. (2020) 4:1–7. doi: 10.1210/JENDSO/BVAA142

69. Haven CJ, Wong FK, van Dam EWCM, van der Luijt R, van Asperen C, Jansen J, et al. A genotypic and histopathological study of a large dutch kindred with hyperparathyroidism-jaw tumor syndrome. *J Clin Endocrinol Metab*. (2000) 85:1449–54. doi: 10.1210/JCEM.85.4.6518

70. Bricaire L, Odou MF, Cardot-Bauters C, Delemer B, North MO, Salenave S, et al. Frequent large germline HRPT2 deletions in a french national cohort of patients with primary hyperparathyroidism. *J Clin Endocrinol Metab*. (2013) 98:E403–8. doi: 10.1210/JC.2012-2789

71. Strande NT, Riggs ER, Buchanan AH, Ceyhan-Birsoy O, DiStefano M, Dwight SS, et al. Evaluating the clinical validity of gene-disease associations: an evidence-based framework developed by the clinical genome resource. *Am J Hum Genet*. (2017) 100:895–906. doi: 10.1016/J.AJHG.2017.04.015

72. Raygada M, Raffeld M, Bernstein A, Miettinen M, Glod J, Hughes MS, et al. Case report of adrenocortical carcinoma associated with double germline mutations in MSH2 and RET. *Am J Med Genet Part a*. (2021) 185:1282. doi: 10.1002/AJMG.A.62099

73. Mallick S, Chakrabarti J, Eschbacher J, Moraitis AG, Greenstein AE, Churko J, et al. Genetically engineered human pituitary corticotroph tumor organoids exhibit divergent responses to glucocorticoid receptor modulators. *Trans Res*. (2023) 256:56–72. doi: 10.1016/j.trsl.2023.01.002

74. Luca E, Zitzmann K, Bornstein S, Kugelmeier P, Beuschlein F, Nölting S, et al. Three dimensional models of endocrine organs and target tissues regulated by the endocrine system. *Cancers (Basel)*. (2023) 15(18):4601. doi: 10.3390/CANCERS15184601

75. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. (2007) 131:861–72. doi: 10.1016/J.CELL.2007.11.019

76. Noltes ME, Sondorp LHJ, Kracht L, Antunes IF, Wardenaar R, Kelder W, et al. Patient-derived parathyroid organoids as a tracer and drug-screening application model. *Stem Cell Rep*. (2022) 17:2518–30. doi: 10.1016/J.STEMCR.2022.09.015

77. Klementieva N, Goliusova D, Krupinova J, Yanvarev V, Panova A, Mokrysheva N, et al. A novel isogenic human cell-based system for men1 syndrome generated by crispr/cas9 genome editing. *Int J Mol Sci*. (2021) 22(21):12054. doi: 10.3390/ijms222112054

78. Even-Zohar N, Metin-Armagan D, Ben-Shlomo A, Sareen D, Melmed S. Generation of isogenic and homozygous MEN1 mutant cell lines from patient-derived iPSCs using CRISPR/Cas9. *Stem Cell Res*. (2023) 69:103124. doi: 10.1016/j.scr.2023.103124

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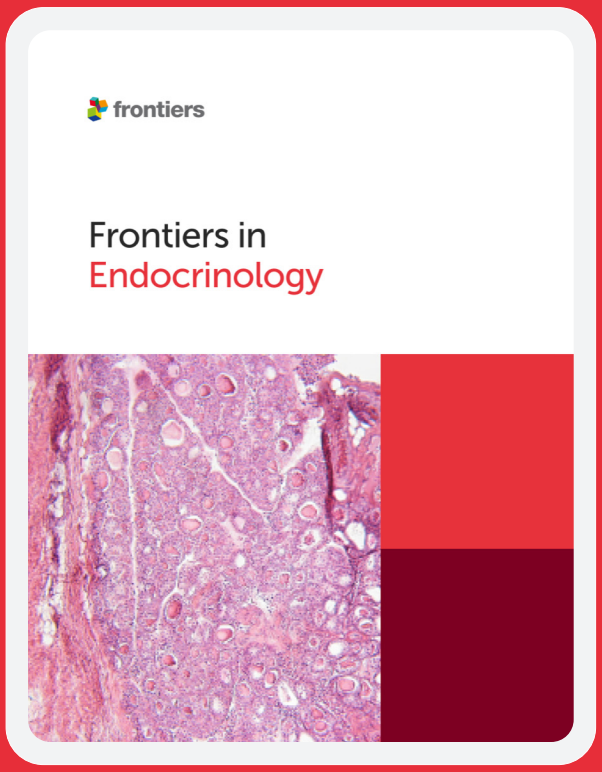

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