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Analysis of the association between mitochondrial dysfunction and common female reproductive endocrinological disorders in a Hungarian cohort

PhD thesis

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Acyl-CoA: Acyl Coenzyme A

AGE: Advanced Glycation End Products

ANOVA: Analysis of variance

AMH: Anti-Müllerian Hormone

ATP: Adenosine triphosphate

BMI: Body mass index

CI: Confidence interval

DAG: Diacylglycerol

DNA: Deoxyribonucleic Acid

ELISA: Enzyme-Linked Immunosorbent Assay

ER: Endoplasmic reticulum

ETC: Electron transport chain

FGF-21: Fibroblast growth factor-21

FSH: Follicle-Stimulating Hormone

GDF-15: Growth differentiation factor-15

GFRAL: GDNF Family Receptor Alpha-Like

GI: Gastrointestinal

GLP-1: Glucagon-like peptide-1

GnRH: Gonadotropin-releasing hormone

HOMA-IR: Homeostatic model assessment of insulin resistance

IGT: Impaired glucose tolerance

IL-6: Interleukin-6

IR: Insulin resistance

IRS: Insulin receptor substrate

LH: Luteinizing Hormone

MASH: Metabolic dysfunction-associated steatohepatitis

MASLD: Metabolic dysfunction-associated steatotic liver disease

MELAS (Mitochondrial Encephalopathy, Lactic Acidosis, and Stroke-like episodes)

MFN1: Mitofusin-1

MFN2: Mitofusin-2

mtDNA: Mitochondrial DNA

mtDNAcn: Mitochondrial DNA copy number

OGTT: Oral glucose tolerance test

OXPHOS: Oxidative phosphorylation

PBS: Phosphate-buffered saline

PCOS: Polycystic ovary syndrome

PCR: Polymerase chain reaction

POI: Premature ovarian insufficiency

PTP1B: Protein tyrosine phosphatase 1B

QUICKI: Quantitative insulin sensitivity check index

ROS: Reactive oxygen species

rRNA: ribosomal RNA

SEM: Standard error of the mean

SHBG: Sex hormone-binding globulin

SNP: Single-nucleotide polymorphisms

T2DM: Type 2 diabetes mellitus

T3: Triiodothyronine

T4: Thyroxine

TGF-β: Transforming Growth Factor Beta

TIA: Transient ischemic attack

TNF-α: Tumour necrosis factor-α

tRNA: transfer RNA

TSH: Thyroid-stimulating hormone

UPR: Unfolded protein response

UV: Ultraviolet

1. INTRODUCTION

1.1. Insulin resistance as a central metabolic disturbance

Insulin resistance (IR) has emerged as one of the most prevalent metabolic abnormalities worldwide and represents a pivotal early step in the development of major non-communicable diseases. It is tightly linked to obesity, type 2 diabetes mellitus (T2DM), metabolic syndrome, metabolic dysfunction-associated steatotic liver disease (MASLD), and several endocrine and reproductive disorders, including polycystic ovary syndrome (PCOS). Sedentary lifestyle, high-calorie diets, and population aging have all contributed to the rapidly increasing burden of IR in recent decades (1, 2). Epidemiological surveys suggest that the prevalence of IR may range between approximately 15–45% in various populations, depending on diagnostic criteria and risk profile (3, 4).

Under physiological conditions, insulin secreted by pancreatic β-cells promotes glucose uptake and storage in skeletal muscle and adipose tissue, and suppresses hepatic glucose production by inhibiting glycogenolysis and gluconeogenesis. It also restrains lipolysis in adipocytes and facilitates anabolism (5). In IR, these actions are only partially effective: hepatic gluconeogenesis and glycogenolysis are inappropriately increased, skeletal muscle glucose uptake is impaired, and adipose tissue exhibits enhanced lipolysis with elevated circulating free fatty acids. The resulting combination of hyperglycaemia, hyperinsulinaemia, and lipotoxicity contributes to systemic metabolic stress and end-organ damage (6).

The etiology of IR is heterogeneous. In many cases, excess adiposity, especially visceral fat accumulation, is the predominant driver. However, genetic defects in the insulin signaling pathway (known as type A insulin resistance), autoantibodies against insulin receptors (type B), drug-induced IR, partial lipodystrophies, chronic stress, and complex post-receptor signaling disorders have also been described (7). At the molecular level, several mechanisms contribute to impairing insulin action:

- overexpression of protein tyrosine phosphatase 1B (PTP1B), which is a negative regulator of insulin receptor phosphorylation (8);
- chronic low-grade inflammation with increased tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and adipokines that interfere with insulin signaling (9, 10);

- oxidative stress, which activates serine/threonine kinases that promote degradation or inhibitory phosphorylation of insulin receptor substrates (IRS) (11);
- endoplasmic reticulum (ER) stress and the unfolded protein response (UPR), which further impair insulin signaling and β-cell survival (12).

Clinically, IR can be assessed by a variety of surrogate markers and indices, including fasting insulin, the homeostasis model assessment of insulin resistance (HOMA-IR), the quantitative insulin sensitivity check index (QUICKI), oral glucose tolerance test (OGTT)-derived indices, and, in research settings, the hyperinsulinemic-euglycemic clamp (13, 14). However, there is no universally accepted gold standard for routine clinical practice. In reproductive endocrinology, IR is often present even when glucose levels remain within the reference range, and careful interpretation of insulin curves, sex hormone-binding globulin (SHBG), and other surrogate markers is required (15, 16).
Taken together, IR can be viewed as a central, upstream pathological state that underlies or amplifies many modern metabolic diseases, including obesity, T2DM, MASLD, atherosclerosis, metabolic syndrome, and female infertility (5, 6).

1.2. Polycystic ovary syndrome as an endocrine-metabolic spectrum disorder

Polycystic ovary syndrome is among the most prevalent endocrine disorders affecting women of reproductive age, impacting an estimated 8–13% of this population globally and serving as the primary cause of anovulatory infertility (17, 18). PCOS is classically characterised by ovulatory dysfunction, clinical and/or biochemical hyperandrogenism, and polycystic ovarian morphology on ultrasound. Beyond its reproductive manifestations, PCOS is associated with IR, central obesity, dyslipidaemia, increased cardiovascular risk, and psychosocial comorbidities, such as anxiety and depression (19, 20).

1.2.1. Etiology, genetic and epigenetic factors

The etiology of PCOS is multifactorial. Family studies, twin concordance data, and genome-wide association studies support a substantial genetic contribution, although currently known genetic variants account for only a minority of the heritability (19, 21). Risk loci include genes involved in gonadotrophin secretion and action, ovarian steroidogenesis, insulin signaling, and adipocyte biology, among others (22, 23). More than 200 single-nucleotide polymorphisms (SNPs) have been implicated in PCOS susceptibility, many in genes regulating androgen synthesis, theca cell function, and hypothalamic-pituitary signaling.

Epigenetic mechanisms and prenatal environmental influences also appear to be important. Maternal obesity, gestational diabetes, smoking, vitamin D deficiency, chronic stress, and hypertensive disorders of pregnancy have been associated with increased risk of PCOS and metabolic dysfunction in offspring, presumably via intrauterine programming of endocrine and metabolic pathways (23). In addition, exposure to endocrine-disrupting chemicals such as bisphenol A may further perturb metabolic and reproductive homeostasis (24). An unhealthy diet, physical inactivity, and subsequent weight gain reinforce the metabolic and endocrine disturbances and may unmask or aggravate the underlying susceptibility.

1.2.2. Diagnostic criteria, phenotypes, and comorbidity burden

Several diagnostic criteria have been proposed for polycystic ovary syndrome (PCOS). The 2003 Rotterdam criteria, which are currently the most widely applied in clinical practice, define PCOS by the presence of at least two of the following three features, after excluding other possible causes: (i) oligo- or anovulation, (ii) clinical and/or biochemical hyperandrogenism, and (iii) polycystic ovarian morphology detected by ultrasound (19). Based on the different combinations of these criteria, four phenotypes (A–D) can be identified. These phenotypes are defined by the presence or absence of three key diagnostic characteristics: oligo-/anovulation (O), clinical and/or biochemical

hyperandrogenism (H), and polycystic ovarian morphology (P), and they differ in their associated metabolic risk profiles (25, 26).

The four phenotypes are defined as follows:

- Phenotype A (classic PCOS): O + H + P
- Phenotype B (non-PCOM classic PCOS): O + H
- Phenotype C (ovulatory PCOS): H + P
- Phenotype D (non-hyperandrogenic PCOS): O + P

Phenotypes A and B are often considered the “classic” or more severe forms, typically associated with higher prevalence of insulin resistance, metabolic dysfunction, and long-term cardiometabolic risk. In contrast, phenotype C presents with preserved ovulatory function despite hyperandrogenism, while phenotype D lacks hyperandrogenism and is sometimes characterized by a milder metabolic profile (25, 26).

This phenotypic variability reflects the complex pathophysiology of PCOS and supports the concept that distinct metabolic and endocrine mechanisms may predominate in different subgroups. Such heterogeneity is particularly relevant when investigating mitochondrial dysfunction and metabolic stress biomarkers, as underlying metabolic burden may vary significantly across phenotypes.

Women with PCOS are at markedly increased risk of impaired glucose tolerance (IGT) and T2DM, with some cohorts reporting IGT in up to 30-40% of affected women. They also have higher rates of metabolic syndrome, MASLD, hypertension, atherogenic dyslipidemia, and endometrial hyperplasia or carcinoma (18, 19). Importantly, low-grade chronic inflammation and oxidative stress are characteristic features that link PCOS with broader cardiometabolic risk (27–29).

1.2.3. Pathophysiological interplay between insulin resistance and hyperandrogenism

The pathophysiology of PCOS reflects the complex interplay between hypothalamic-pituitary-ovarian axis dysregulation and metabolic abnormalities, particularly IR. The increased frequency and amplitude of gonadotropin-releasing hormone (GnRH) pulses from the hypothalamus favor luteinizing hormone (LH) secretion over follicle-stimulating hormone (FSH) secretion. Elevated LH stimulates the ovarian theca cells to increase androgen production, while relatively low or normal FSH contributes to arrested follicular development and anovulation (30).

Hyperinsulinemia acts synergistically with LH to further enhance androgen biosynthesis in the ovary and adrenal glands and suppress hepatic production of SHBG, thereby increasing free, bioactive androgen levels (19). Conversely, hyperandrogenism promotes visceral fat accumulation and worsens IR, partly through effects on adipocyte differentiation and inflammatory signaling. Adipose tissue dysfunction, characterised by altered secretion of adipokines and pro-inflammatory cytokines, further impairs insulin action in the liver and muscle and amplifies systemic inflammation. These reciprocal interactions create a self-perpetuating "vicious circle" linking PCOS, abdominal adiposity, IR, and low-grade inflammation (29, 31).

1.3. Premature ovarian insufficiency as a complex reproductive-metabolic disorder

Premature ovarian insufficiency (POI) is a heterogeneous clinical condition characterized by the loss of normal ovarian activity before the age of 40 years, leading to menstrual irregularities, hypoestrogenism, and reduced fertility potential (32, 33). In addition to its reproductive consequences, POI is associated with long-term health risks, including osteoporosis, cardiovascular disease, metabolic disturbances, and impaired quality of life (34, 35). From a pathophysiological perspective, POI represents a final common pathway of accelerated ovarian aging and follicular depletion arising from diverse etiological mechanisms, encompassing genetic abnormalities, autoimmune processes, iatrogenic injury, and environmental influences (36, 37)

The causes of POI are multifactorial and remain unidentified in a substantial proportion of cases. Established contributors include genetic abnormalities affecting ovarian development and folliculogenesis, autoimmune-mediated ovarian damage, iatrogenic injury following chemotherapy, radiotherapy, or ovarian surgery, as well as environmental and lifestyle-related factors (38). Increasing evidence suggests that metabolic disturbances and systemic stress responses may further modulate ovarian vulnerability and disease progression. Similar to insulin resistance (IR) and polycystic ovary syndrome (PCOS), POI should therefore be viewed not as an isolated ovarian disorder but as part of a broader endocrine-metabolic spectrum influenced by genetic predisposition, immune dysregulation, environmental exposures, and medical interventions (39, 40).

At the cellular level, oxidative stress has emerged as a central mechanism linking metabolic dysfunction to ovarian aging. Excessive generation of reactive oxygen species (ROS), particularly in the setting of impaired antioxidant defense, contributes to mitochondrial damage, altered energy production, and activation of apoptotic pathways. Oocytes are especially susceptible to oxidative injury due to their high mitochondrial content and limited capacity for cellular renewal. Accumulation of mitochondrial DNA damage, disruption of mitochondrial dynamics, and declining mitochondrial function are therefore thought to play a critical role in follicular atresia and premature depletion of the ovarian reserve (41, 42).

Understanding the molecular and metabolic mechanisms underlying POI is essential for improving diagnostic precision and identifying novel therapeutic targets. In particular, elucidating the contribution of mitochondrial dysfunction and oxidative stress may help to integrate POI into a unified framework of IR-associated reproductive disorders, alongside PCOS, and provide a rationale for the investigation of mitochondrial biomarkers in affected women.

1.4. Mitochondrial dysfunction in carbohydrate metabolism and female reproduction

Mitochondria are central organelles in eukaryotic cells that are responsible not only for ATP production through oxidative phosphorylation (OXPHOS), but also for fatty acid

oxidation, intermediary metabolism, apoptosis, steroidogenesis, and redox homeostasis (43, 44). They contain their own circular mitochondrial DNA (mtDNA), which encodes 13 essential subunits of the respiratory chain, 22 transfer RNAs (tRNAs), and 2 ribosomal RNAs (rRNAs). The inner membrane of the mitochondrion contains complexes I–IV of the electron transport chain (ETC) and ATP synthase (complex V), which collectively perform proton pumping and ATP synthesis. During this process, some electrons leak from the ETC and react with oxygen, leading to the formation of ROS (45).

Under normal conditions, cellular antioxidant systems balance ROS generation and prevent oxidative damage. If this balance is disrupted, oxidative stress damages lipids, proteins, and nucleic acids, leading to mitochondrial dysfunction. In the context of metabolic diseases, mitochondrial dysfunction is characterized by reduced oxidative capacity and ATP production, increased ROS generation, accumulation of lipid intermediates, altered mitochondrial dynamics (fusion/fission), and impaired mitophagy (46–49).

In pancreatic β-cells, proper mitochondrial function is essential for glucose sensing and insulin secretion. Hyperglycemia, lipotoxicity, and chronic inflammation all compromise mitochondrial integrity, leading to β-cell dysfunction and apoptosis, thereby promoting progression from IR to overt T2DM (50, 51). Mitochondrial dysfunction in the liver and skeletal muscle similarly contributes to IR by reducing oxidative capacity and allowing the accumulation of lipid metabolites that interfere with insulin signaling (52). Advanced glycation end products (AGEs) formed in chronic hyperglycemia further exacerbate oxidative stress by impairing antioxidant enzymes and inducing inflammatory signaling, aggravating mitochondrial damage (53).

From a reproductive perspective, oocytes are among the cells with the highest number of mitochondria in the human body. The number, distribution, and functional competence of mitochondria are critical determinants of oocyte maturation, fertilization potential, and early embryo development (54, 55). Experimental studies have shown that targeted disruption of mitochondrial fusion proteins Mitofusin-1 (MFN1) or Mitofusin-2 (MFN2) in oocytes leads to altered mitochondrial dynamics, accelerated follicular depletion, increased apoptosis, telomere shortening, and infertility in mouse models (56). In women with PCOS, granulosa cells exhibit elevated intracellular ROS levels, impaired

mitochondrial function, and increased apoptosis, which may contribute to reduced oocyte quality and subfertility (57, 58).

At the genomic level, numerous mtDNA variants, including point mutations in mitochondrial tRNAs, polymorphisms in the D-loop control region, and large-scale deletions, have been associated with the development of T2DM, IR, and PCOS (29, 59–62). Alterations in mtDNA copy number (mtDNAcn) represent another important facet of mitochondrial dysfunction. Several studies have reported decreased mtDNAcn in patients with T2DM, prediabetes, and PCOS, as well as in women undergoing in vitro fertilization, where lower mtDNAcn in oocytes has been linked to reduced fertilisation rates and embryo quality (63, 64). Large-scale mtDNA deletions, particularly when present in a multiplex pattern, reflect cumulative damage to the mitochondrial genome and have been described in both primary mitochondrial disorders and in secondary mitochondrial dysfunction characterized by increased oxidative stress (65–67).

Together, these data support the concept that mitochondrial dysfunction is not only a consequence but also a cause of metabolic and reproductive disorders, placing mitochondria at the intersection of insulin resistance, polycystic ovary syndrome, and female fertility. In contrast to circulating stress-responsive mitokines, such as GDF-15, mtDNA deletions are DNA-based biomarkers that reflect cumulative structural damage to the mitochondrial genome.

1.5. Circulating mitochondrial biomarkers, with an emphasis on growth differentiation factor 15 (GDF-15)

Given the invasive nature and limited availability of tissue biopsies, there is increasing interest in circulating biomarkers that reflect mitochondrial function or stress. Several classes of blood-based mitochondrial biomarkers have been proposed, including:

- classical metabolic intermediates (lactate, pyruvate, acylcarnitines, amino acids);
- DNA-based markers (mtDNA copy number, mtDNA deletions, and mutations);
- and stress-induced cytokines, often referred to as “mitokines”, such as fibroblast growth factor-21 (FGF-21) and growth and differentiation factor-15 (GDF-15) (68–70).

GDF-15 is a divergent member of the transforming growth factor-β (TGF-β) superfamily, produced at low levels by many cell types under basal conditions but markedly upregulated in response to diverse forms of cellular stress, including inflammation, hypoxia, oxidative damage, tissue injury, oncogenic transformation, and mitochondrial dysfunction (71–74). Pregnancy is the only physiological state known to be associated with sustained, marked elevations of circulating GDF-15 (75).

Clinically, GDF-15 has been most extensively studied in cardiovascular disease, where higher plasma levels predict adverse outcomes in heart failure, coronary artery disease, ST-elevation myocardial infarction, and non-ST-elevation acute coronary syndromes (76–79). Elevated GDF-15 has also been reported in chronic kidney disease, neurodegenerative disorders, chronic obstructive pulmonary disease, pulmonary embolism, autoimmune diseases, infections, and a wide range of malignancies (80–83).

Importantly, GDF-15 and FGF-21 have emerged as sensitive biomarkers of primary mitochondrial diseases, such as MELAS and Leigh syndrome, where very high circulating levels reflect severe mitochondrial stress and respiratory chain dysfunction (84–87). In metabolic conditions, including obesity, MASLD/MASH, and metabolic syndrome, elevations in GDF-15 appear to represent a compensatory response aimed at mitigating mitochondrial stress and lipid accumulation (88–90).

Beyond its role as a damage signal, GDF-15 exerts functional effects on appetite and energy homeostasis via its specific receptor GFRAL (glial cell line-derived neurotrophic factor family receptor alpha-like), which is selectively expressed in the area postrema and nucleus tractus solitarius of the brainstem. Binding of GDF-15 to GFRAL and its co-receptor RET induces anorexigenic signalling, reduces food intake and body weight, and improves glucose tolerance in preclinical models (91–94). In humans, metformin therapy consistently increases circulating GDF-15 levels, and GDF-15 has been implicated as a mediator of the weight-reducing and appetite-suppressing effects of metformin (95–98).

In the context of glucose metabolism, GDF-15 levels are elevated in obesity, prediabetes, and T2DM, correlate with indices of IR and cardiometabolic risk, and may serve as a predictor of incident diabetes and metabolic deterioration (99–102). Exercise and caloric restriction modulate GDF-15 levels in complex ways, and it has been proposed that GDF-15 may form part of a broader adaptive mitochondrial stress response in metabolic syndrome (103–105).

Data on GDF-15 in PCOS are still limited. Available studies suggest that GDF-15 levels may be altered in PCOS and that a relative "GDF-15 deficiency" could contribute to the pronounced tendency to weight gain and IR, particularly in lean PCOS phenotypes (106–108). Given its dual role as a mitokine and as a regulator of energy balance, GDF-15 is an attractive candidate biomarker at the interface of IR, mitochondrial stress, and female reproductive dysfunction.

1.6. Knowledge gaps and study rationale

The literature reviewed above highlights several converging lines of evidence:

- Insulin resistance is a central driver of both metabolic and reproductive morbidity, particularly in women with PCOS and related endocrine disorders.
- Mitochondrial dysfunction and oxidative stress are common denominators of IR, T2DM, obesity, metabolic syndrome, and PCOS.
- Oocyte quality, ovarian aging, and female fertility are tightly coupled to mitochondrial function and mtDNA integrity.
- Circulating mitokines, and especially GDF-15, together with mtDNA-based markers such as mtDNA copy number and mtDNA deletions, represent promising tools to capture systemic and tissue-level mitochondrial stress non-invasively.

However, several important knowledge gaps remain:

1. **Integrated assessment across insulin resistance-related reproductive phenotypes.**

   Most studies have examined GDF-15 or mtDNA alterations in isolation, focusing on cardiometabolic disease, primary mitochondrial disorders, or PCOS as a single entity. In contrast, few studies have systematically compared IR, PCOS coexisting with IR, and POI coexisting with IR within the same clinical setting using both GDF-15 measurement and mtDNA deletion analysis.

2. **Link between mitochondrial stress markers and detailed endocrine-reproductive profiles.**

   The relationship between GDF-15, mtDNA deletions, and key reproductive hormones (e.g., AMH, gonadotrophins, sex steroids), as well as markers of

ovarian reserve and response, has not been comprehensively characterized in women across the spectrum of insulin resistance.

3. **Interactions with thyroid function and multiorgan symptom burden.**
   Thyroid hormones strongly influence mitochondrial biogenesis and oxidative metabolism, yet the interplay between thyroid hormones, GDF-15, and mtDNA integrity in IR and in women with PCOS or POI remains largely unexplored. Furthermore, it is unclear to what extent multiorgan symptom complexes (neuromuscular, autonomic, gastrointestinal, or neuropsychiatric complaints) in women with IR and PCOS or POI reflect an underlying mitochondrial contribution captured by these biomarkers.

4. **Defining mitochondrial phenotypes within IR-associated reproductive disorders.**
   It is not yet known whether distinct mitochondrial phenotypes can be delineated among women with IR, IR-PCOS, and IR-POI, based on combined patterns of GDF-15 levels, mtDNA deletions, and clinical and endocrine features, nor whether such phenotypes may have implications for prognosis or treatment stratification.

The overarching rationale of this dissertation is to address these gaps by integrating GDF-15 as a circulating marker of mitochondrial stress with mtDNA deletion analysis and comprehensive metabolic, endocrine, and reproductive phenotyping in women with IR and IR-associated reproductive disorders (PCOS and POI). By doing so, this work aims to advance our understanding of the mitochondrial contribution to female metabolic–reproductive pathology and to lay the groundwork for future mitochondria-informed diagnostic and therapeutic strategies in this patient population. Beyond its well-established metabolic consequences, insulin resistance may represent a state of accelerated biological aging, with a particular impact on mitochondrial integrity and reproductive function. However, the role of mitochondrial stress markers in accelerating reproductive aging across IR-related phenotypes has not yet been systematically investigated.

To the best of our knowledge, this is the first study to integrate circulating mitokine profiling and mitochondrial DNA deletion analysis to investigate insulin resistance-related reproductive phenotypes, including IR, IR-PCOS, and IR-POI, in a single clinically characterized cohort.

2. OBJECTIVES

The aims of our study were:

1. To determine the prevalence, pattern, and clinical relevance of mitochondrial DNA (mtDNA) deletions and elevated plasma GDF-15 levels in women with insulin resistance (IR), IR-PCOS, and IR-POI, compared with healthy controls.
   This aim establishes the fundamental mitochondrial stress profile underlying these endocrine-metabolic disorders.
2. To investigate the relationship between GDF-15, mtDNA deletions, and metabolic burden, including BMI, reactive hyperinsulinemia, OGTT-derived insulin dynamics, and HOMA index, as well as the required intensity of metabolic therapy (metformin and GLP-1 receptor agonists).
   The objective is to evaluate whether GDF-15 functions as a quantitative biomarker of metabolic stress and treatment demand.
3. To examine the associations of GDF-15 and mtDNA deletions with key reproductive and thyroid hormone parameters (AMH, FSH, LH, estradiol, testosterone, prolactin, TSH, free T4).
   The purpose of this aim is to investigate the endocrine-mitochondrial regulatory axis in insulin resistance-related disorders.
4. To assess the link between mitochondrial dysfunction (mtDNA deletions and/or elevated GDF-15) and markers of reproductive aging, including AMH levels, AMH/FSH ratio, and age-dependent ovarian reserve trajectories.
   The goal is to determine whether mitochondrial dysfunction contributes to accelerated reproductive aging in women with insulin resistance.
5. To characterize multisystem clinical involvement (musculoskeletal, gastrointestinal, neuropsychiatric, autoimmune, and additional endocrine manifestations) in relation to GDF-15 levels and mtDNA deletion status, thereby defining the systemic phenotype associated with mitochondrial dysfunction in IR-related endocrine disorders.
   This objective examines the extent to which IR, PCOS, and POI exhibit a broader “mitochondrial phenotype”.

3. METHODS

3.1. Study design and patient population

This retrospective observational study was conducted at the Department of Obstetrics and Gynecology and the Institute of Genomic Medicine and Rare Diseases, Semmelweis University, Budapest. Female patients were recruited between 2022 and 2024 and comprise the Polycystic Ovary Syndrome, Mitochondrial Dysfunction, Obesity, Insulin Resistance, Infertility (POMODORI) cohort (ClinicalTrials.gov identifier: NCT06167135). Three patients were recruited from the NEPSYBANK of the Institute of Genomic Medicine and Rare Diseases, as IR and PCOS were also present in these patients with known mitochondrial abnormalities (109, 110).

Eligible participants were women of reproductive age (20-45 years) referred for evaluation of insulin resistance (IR), infertility, and associated reproductive endocrine disorders, including polycystic ovary syndrome (PCOS) and premature ovarian insufficiency (POI). Most patients presented with symptoms or complaints affecting multiple organ systems, raising the possibility of systemic metabolic or mitochondrial involvement.

Inclusion criteria comprised:

(I) confirmed insulin resistance, with or without a diagnosis of PCOS or POI;
(II) age between 20 and 45 years;
(III) provision of written informed consent for biochemical and molecular genetic testing.

Exclusion criteria included pregnancy, active infection, malignancy, acute inflammatory disease at the time of sampling, and the use of hormonal or metabolic therapy within three months prior to enrollment. Patients who were later found to be pregnant at the time of blood sampling were excluded because plasma GDF-15 levels are physiologically elevated during pregnancy.

The final study population consisted of 81 patients: 49 with isolated IR, 19 with IR and PCOS, and 13 with IR and POI. The mean age of patients was 35.38 ± 6 years (95% CI: 34.23-36.53). A control group of 41 age-matched, healthy women without IR, PCOS,

POI, or other metabolic disorders and with normal body mass index (BMI 20-25 kg/m²) was included. Pregnancy was excluded in all control participants at the time of sampling.

The study was conducted in accordance with the Declaration of Helsinki and approved by the Hungarian National Centre for Public Health Ethics Committee (approval number: 15672-6/2022/ECIG). All participants received pre-test clinical genetic counseling and participated voluntarily. The POMODORI cohort is a structured clinical biobank that aims to enable the integration of metabolic, endocrine, and mitochondrial investigations in women with insulin resistance and related reproductive disorders. Biological samples (peripheral blood, plasma, and urine epithelial cells) were collected according to standardised protocols. To ensure sample integrity, the time interval between collection and processing or long-term storage did not exceed two hours. Importantly, clinical phenotyping and laboratory assessments, including metabolic and hormonal parameters, were performed at the same time as the collection of biological samples, ensuring that the timing of biomarker measurements and clinical data was aligned in both patients and healthy controls. Samples were then stored under controlled conditions for future analyses. In parallel, detailed clinical, metabolic, endocrine, and multisystem phenotyping was performed to enable genotype-phenotype correlations to be established. This dissertation is the first systematic analysis to be conducted within this cohort.

3.2. Clinical assessment and laboratory investigations

All participants underwent a detailed clinical evaluation, including a structured clinical questionnaire and physical examination. The questionnaire was used to identify cardiovascular, gastrointestinal, neuromuscular, neurological, visual, and auditory symptoms, exercise intolerance (reduced ability to perform physical activity at levels expected for an individual of comparable age and general health status), thermoregulatory abnormalities, neuropsychiatric manifestations, autoimmune symptoms, and other endocrine conditions, suggestive of multisystem involvement, including features previously associated with mitochondrial dysfunction.

Routine laboratory investigations included oral glucose tolerance test (OGTT) results (fasting, 60-minute, and 120-minute serum glucose and insulin levels) and calculation of the homeostasis model assessment of insulin resistance (HOMA-IR).

Insulin resistance was defined according to the guidelines of the Hungarian Society for Obstetric and Gynecological Endocrinology: insulin levels >10 mU/L at 0 minutes, >50 mU/L at 60 minutes, and >30 mU/L at 120 minutes during OGTT. The diagnosis of PCOS was established using the 2003 Rotterdam criteria. Premature ovarian insufficiency was defined as reduced ovarian reserve in women under 40 years of age, indicated by an anti-Müllerian hormone (AMH) level below 1.0 ng/mL.

Hormonal parameters included serum AMH, follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol, progesterone, prolactin, total testosterone, thyroid-stimulating hormone (TSH), free thyroxine (T4), free triiodothyronine (T3), and vitamin D3. AMH, FSH, LH, estradiol, and prolactin were measured between days 2 and 4 of the menstrual cycle, while progesterone was assessed between days 22 and 24, where applicable. Hormone levels in patients receiving oral contraceptive therapy during the sampling period were not incorporated into the analysis.

3.3. Sample collection and DNA isolation

Peripheral blood, urine epithelial cell samples, and plasma were collected simultaneously from each participant. Plasma samples were processed and stored at -80°C within one hour of collection to preserve protein integrity.

DNA was isolated from both peripheral blood leukocytes and urine epithelial cells using the DNeasy® Blood & Tissue Kit (QIAGEN GmbH, Hilden, Germany), following the manufacturer's protocol. Urine epithelial cells were used as a non-invasive alternative tissue for mitochondrial DNA (mtDNA) analysis, as previous studies have demonstrated good concordance with skeletal muscle heteroplasmy levels (111).

For urine samples, DNA was isolated from 100 mL of freshly collected urine. Samples were centrifuged at 1000×g for 10 minutes, washed with phosphate-buffered saline, and

centrifuged again at 1000×g for 10 minutes. DNA was extracted from the resulting cell pellet. DNA concentration and purity were determined spectrophotometrically using absorbance at 260 nm and the A260/A280 ratio.

3.4. Analysis of mitochondrial DNA deletions

Mitochondrial DNA deletion analysis was performed in parallel on blood- and urine-derived DNA samples from all participants. Long-range polymerase chain reaction (PCR) was used to detect the 4977-bp "common deletion" as well as multiple mtDNA deletions.

PCR amplification targeted an approximately 8600-bp region of mtDNA spanning nucleotide positions 8232–16,496. Reactions were performed in a 20 µL volume using Phusion DNA polymerase (Thermo Fisher Scientific Inc., Waltham, MA, USA), GC-rich reaction buffer, 200 µM dNTPs, 200 pM forward (5′-TAAAAATCTTTGAAATAGGGC-3′) and reverse (5′-CGGATACAGTTTTCACTTTAGCT-3′) primers, and approximately 30 ng of template DNA.

The PCR cycling conditions were as follows: initial denaturation at 98°C for 30 seconds; 35 cycles of 98°C for 10 seconds, 63°C for 10 seconds, and 72°C for 3 minutes; followed by final extension at 72°C for 7 minutes. PCR products were separated on 1% high-resolution agarose gels and visualized relative to a 1 kb molecular weight marker.

The presence of mtDNA deletions was defined by the detection of additional smaller bands alongside the full-length wild-type fragment. Heteroplasmy ratios were quantified using Quantity One® 1-D Analysis Software (version 4.6.3; Bio-Rad Imaging Systems, Hercules, CA, USA) and ImageJ (version 1.54i). In this study, mtDNA deletions detected by long-range PCR were used as surrogate markers of mitochondrial dysfunction.

Long-range PCR-based detection of mtDNA deletions is a well-established qualitative screening method in clinical mitochondrial diagnostics. Although it does not provide next-generation sequencing-level resolution, it reliably identifies large-scale deletions

present above low-level heteroplasmy thresholds. The parallel analysis of blood- and urine-derived DNA further reduces the likelihood of tissue-specific artefacts.

3.5. Measurement of plasma GDF-15

Plasma concentrations of growth differentiation factor-15 (GDF-15) were measured using a commercially available Human GDF-15 ELISA kit (Thermo Fisher Scientific, BMS2258) according to the manufacturer's instructions. All samples were analyzed in duplicate, and concentrations were calculated using a seven-parameter logistic calibration curve. Age- and sex-specific reference ranges and cut-off values were applied based on a large meta-analysis involving approximately 20,000 individuals (112). These reference values were used to interpret GDF-15 concentrations in both patient and control groups.

3.6. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM), unless otherwise specified. Statistical analyses were performed using Python (version 3.10.5) with the SciPy library (version 1.9.3).

Comparisons between two groups were conducted using the Mann-Whitney U test, while comparisons among multiple groups were performed using the Kruskal-Wallis test followed by Dunn's post hoc analysis. Hormonal parameters were additionally analyzed using one-way analysis of variance (ANOVA) with post hoc testing, following assessment of normality using the Shapiro-Wilk test.

Correlations between variables were assessed primarily using Pearson's correlation coefficient. Spearman's rank correlation was also examined to account for potential non-linear monotonic associations; however, as results were consistent, Pearson's correlations are reported. Linear regression models were used for visualization and predictive analyses. Multiple regression analyses were performed to assess associations between

GDF-15 levels and clinical or biochemical parameters, adjusting for age and BMI as potential confounders.

Differences in proportions were evaluated using the chi-square test. A p-value of <0.05 was considered statistically significant. As this was a retrospective observational study, no formal sample size calculation was performed. Subgroup analyses stratified by mtDNA deletion status were predefined as exploratory and are interpreted as hypothesis-generating.

4. RESULTS

4.1. In patients with IR, levels of plasma GDF-15 and the presence of mtDNA deletions show a significant increase

The mean plasma GDF-15 level was significantly higher in patients than in the control group (1,213.6 ± 83.6 pg/mL, 95% CI: 1,047.2–1,379.9 vs. 572.8 ± 82.8 pg/mL, 95% CI: 405.0–740.7; $p < 0.001$) (see Figure 1A). Receiver operating characteristic (ROC) curve analysis demonstrated adequate sensitivity and specificity of GDF-15 in distinguishing patients from healthy controls (Figure 2). Within the patient cohort, GDF-15 levels did not differ according to mtDNA deletion status. Mean concentrations were 1187.6 pg/mL (95% CI: 1063.5–1311.7) in patients without deletions and 1230.6 pg/mL (95% CI: 1003.6–1457.4) in those harbouring mtDNA deletions ($p = 0.877$) (Figure 1B). Similarly, no significant differences were observed between patients with single versus multiple mtDNA deletions ($p = 0.552$) (Figure 1B). The distribution of mtDNA deletions was similar between patients with normal and elevated GDF-15 plasma levels. MtDNA deletions were detected in 57% of patients with normal GDF-15 levels. Among patients with elevated GDF-15, 55% harboured mtDNA deletions, while 45% were deletion-negative. Additionally, GDF-15 plasma levels exhibited a modest positive correlation with patient age ($R^2 = 0.0558$), consistent with prior studies (Figure 1C) (109).

A comparison of plasma GDF-15 levels across different insulin resistance-related clinical subgroups and healthy controls is shown in Figure 1E. The highest mean GDF-15 concentrations were observed in the IR-only subgroup (1,343.2 ± 105.9 pg/mL, 95% CI: 1,131.0–1,555.4; $p < 0.001$), followed by the IR-POI subgroup (1,207.5 ± 84.9 pg/mL, 95% CI: 1,037.8–1,377.3; $p < 0.001$). Lower, yet still significantly increased, levels were detected in the IR-PCOS subgroup (899.0 ± 131.1 pg/mL, 95% CI: 623.6–1,174.4; $p = 0.003$). A significant difference in GDF-15 levels was observed when compared directly between subgroups (Figure 1E). Specifically, the IR-only subgroup exhibited higher levels than both the IR-PCOS and IR-POI subgroups ($p = 0.011$ and $p = 0.043$) (109).

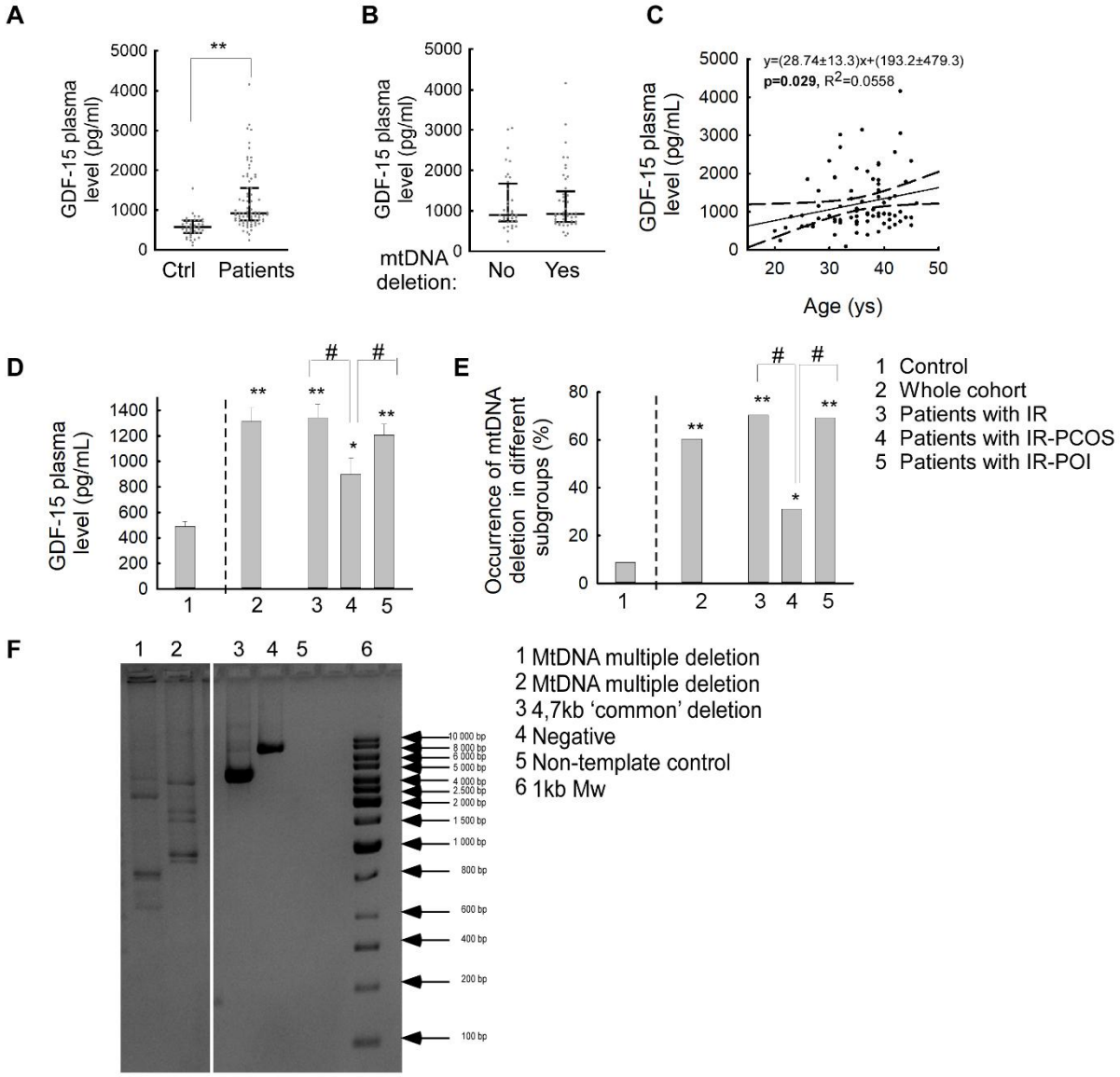

Figure 1. Plasma GDF-15 levels and mtDNA deletions in insulin-resistant patients. (A) Plasma GDF-15 levels in patients and controls. (B) GDF-15 levels stratified by mtDNA deletion status. (C) Correlation between GDF-15 and age. (D) Prevalence of mtDNA deletions in patient subgroups. (E–F) Representative electrophoresis images of mtDNA deletions (109).

The mean plasma GDF-15 levels (expressed as pg/mL) in subgroups of patients with insulin resistance (IR) only and with both IR and polycystic ovary syndrome (PCOS) are shown in the table below. The data are presented as mean ± standard error of the mean (SEM). Significant differences are indicated by the following symbols: **: $p < 0.001$; *: $p < 0.05$; #: $p < 0.05$ (Kruskall–Wallis Dunn). (E) Occurrence of mtDNA deletions in the control group (n = 41) (1), the whole patient group (n = 81) (2), patients with IR only (n = 62) (3) and patients with both IR and PCOS (n = 19) (mean ± SEM; ** $p < 0.001$, * $p < 0.05$, # $p < 0.05$, Kruskal–Wallis Dunn). (F) Representative electrophoresis picture of mtDNA deletions analysed by long-range PCR (Mw: molecular weight marker (1000 bp)).

Interestingly, the prevalence of mtDNA deletions across clinical subgroups largely mirrored the pattern observed for mean plasma GDF-15 levels. In the overall patient cohort, mtDNA deletions were detected in 61.5% of cases, comprising 10.4% of single deletions and 51.1% of multiple deletions (see Figure 1D). The highest prevalence of deletions was observed in the IR-only subgroup (70.3%), followed by the IR-POI subgroup (69.3%). By contrast, the IR-PCOS subgroup exhibited a markedly lower frequency of mtDNA deletions (31.5%), with only multiple deletions detected. It is also notable that multiple mtDNA deletions were present in a small proportion (8.7%) of healthy controls (Figure 1D).

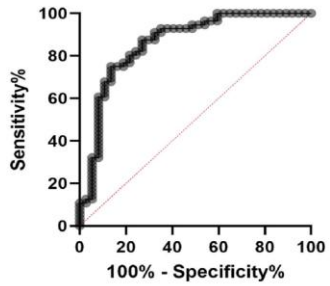

Figure 2: The receiver operating characteristic (ROC) curve analysis based on the GDF-15 results of our patients and the healthy control group (109).

4.2. Elevated GDF-15 is strongly correlated with reactive hyperinsulinemia

The relationship between plasma GDF-15 levels and mtDNA deletion was examined in relation to key metabolic parameters measured at the time of sampling. These analyses were restricted to patients who underwent a standard 75 g oral glucose tolerance test (OGTT). Serum glucose and insulin levels were assessed at 0, 60, and 120 minutes, and the response curves were compared between patients with normal and elevated plasma GDF-15 levels. No significant differences were observed in blood glucose concentrations at any OGTT time point between the two subgroups (Figure 3A). However, serum insulin levels differed significantly: patients with elevated GDF-15 exhibited higher insulin

concentrations at both 60 and 120 minutes (normal GDF-15: 60 minutes, 90.38 ± 58.63 µU/mL; 120 minutes, 59.87 ± 37.92 µU/mL; elevated GDF-15: 60 minutes, 130.45 ± 90.54 µU/mL; 120 minutes, 88.67 ± 39.65 µU/mL) (Figure 3B).

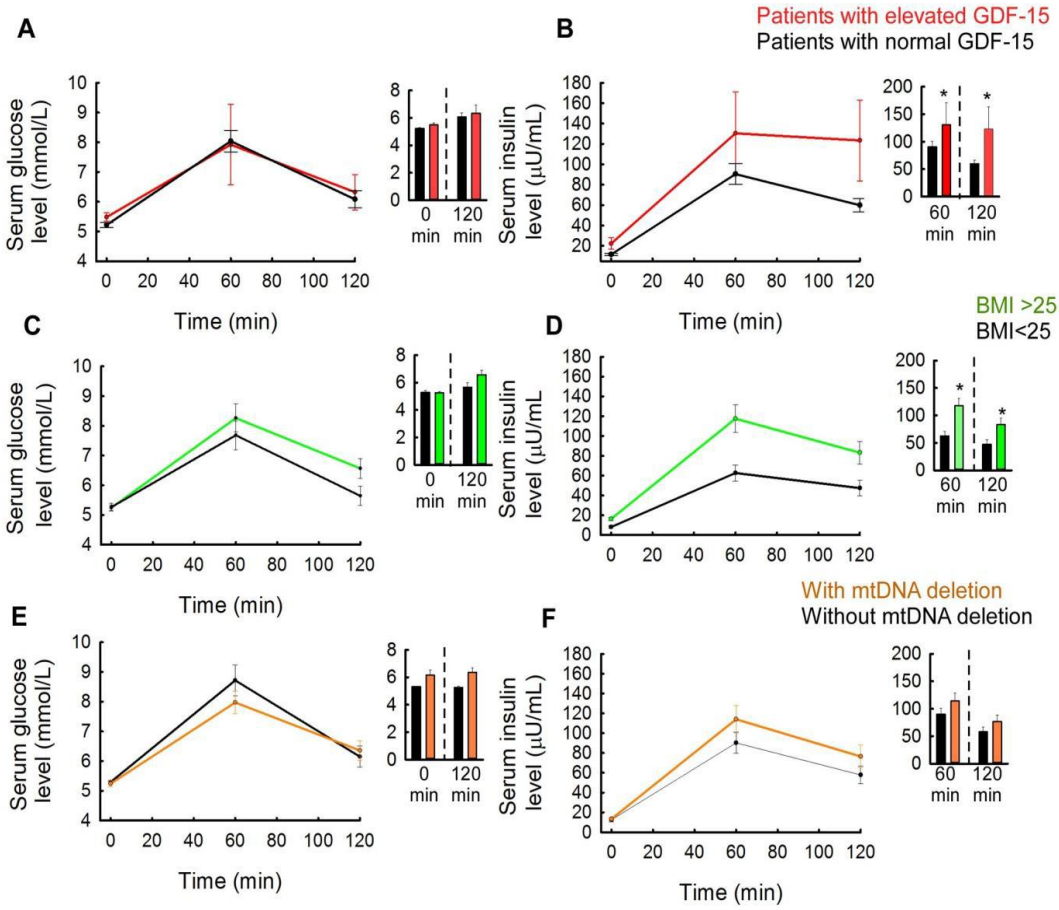

Figure 3. Associations between GDF-15, glucose, insulin, BMI, and mtDNA deletions during OGTT. Panels A-F correspond to glucose and insulin responses stratified by GDF-15 level, BMI, and mtDNA deletion status (109).

Serum glucose levels in patients with elevated and normal GDF-15 levels, respectively, as determined by an oral glucose tolerance test involving 75 g of glucose (measured at 0, 60, and 120 minutes)(A). Patients with normal GDF-15 levels: N = 53; patients with elevated GDF-15 levels (N = 8). (B) Serum insulin levels of patients with elevated and normal GDF-15 levels based on the results of an oral glucose tolerance test involving 75 g of glucose (measured at 0, 60, and 120 minutes; patients with normal GDF-15 levels: N = 53; patients with elevated GDF-15 levels: N = 8). N = 53; patients with elevated GDF-15 levels: N = 8). (C) Serum glucose levels of patients with high and normal BMI, respectively, based on the results of the oral glucose tolerance test with 75 g of glucose (measured at minutes 0, 60, and 120). Patients with normal BMI (<25 kg/m²): N = 36; patients with elevated BMI (≥25 kg/m²): N = 36. (D) Serum insulin levels of patients with high and normal BMI, respectively, based on the results of the oral

glucose tolerance test with 75 g of glucose (measured at minutes 0, 60, and 120). Patients with normal BMI (<25 kg/m²): N = 25. Patients with elevated BMI (≥25 kg/m²): N = 36. N = 25; patients with elevated BMI (≥25 kg/m²): N = 36). (E) Serum glucose levels in patients with and without mtDNA deletions, respectively, based on the results of an oral glucose tolerance test involving 75 g of glucose (measured at minutes 0, 60, and 120). Patients without mtDNA deletion: N = 23; patients with mtDNA deletion: N = 38). (F) Serum insulin levels in patients with and without mtDNA deletions, based on the results of an oral glucose tolerance test involving 75 g of glucose, were measured at 0, 60, and 120 minutes. Patients without mtDNA deletion: N = 23; patients with mtDNA deletion: N = 38) (*: $p < 0.05$, paired t-test).

Consistently, patients with elevated GDF-15 levels had significantly higher HOMA index values than those with normal levels (2.9 ± 2.5 [95% CI: 2.2–3.5] vs. 4.4 ± 2.7 [95% CI: 2.4–6.4], $p < 0.05$). Due to the strong correlation between elevated GDF-15 levels and a higher BMI, the patients were divided into two groups based on BMI: <25 kg/m² and ≥25 kg/m². This was done to determine whether reactive hyperinsulinemia was driven by BMI rather than GDF-15 levels. Within the subgroup with an elevated BMI, a non-significant trend towards higher glucose levels at 120 minutes was observed ($p = 0.07$). The mean glucose values at 120 minutes were 5.64 ± 1.20 mmol/L for patients with a normal BMI and 6.54 ± 1.66 mmol/L for patients with an elevated BMI. However, no significant differences were detected between the BMI-defined subgroups at any OGTT time point (Figure 3C).

Fasting mean insulin levels were significantly increased in patients with elevated BMI ($p < 0.05$). A similar pattern was observed in the subgroup with elevated plasma GDF-15 levels and a BMI <25 (60 min: 62.62 ± 30.28 U/mL; 120 min: 47.41 ± 29.43 U/mL), as well as in patients with both elevated GDF-15 levels and high BMI (60 min: 117.46 ± 69.53 U/mL; 120 min: 83.16 ± 37.03 U/mL). However, there was no significant difference in serum insulin levels at 60 and 120 minutes between the two groups (Figure 3D).

When insulin values at 60 minutes were compared between the high-BMI and elevated GDF-15 subgroups, the mean insulin level was slightly higher in the GDF-15 subgroup, but this difference was not statistically significant ($p = 0.933$). Therefore, it remains unclear whether the observed difference is attributable to elevated GDF-15 levels or to the higher BMI associated with the condition (Figure 3B, D). Finally, two patient

subgroups were created based on the presence or absence of single or multiple mitochondrial DNA deletions (Figures 2E and 2F). No significant difference in blood glucose levels was observed between these groups, and the 60-minute glucose level was even lower in the subgroup with mtDNA deletions than in those without such deletions (Figure 3E). However, a slight increase in insulin levels at both 60 and 120 minutes was noted in the subgroup with mtDNA deletions compared with the subgroup without deletions (Figure 3F).

4.3. Relationship Between Plasma GDF-15, Body Mass Index, and Age

The mean plasma levels of GDF-15 were analysed and compared with healthy controls across predefined body mass index (BMI) categories (<20, 20–24.9, 25–29.9, 30–34.9, and >35 kg/m²). While GDF-15 levels in patients with a BMI below 20 kg/m² were comparable to those observed in the control group, plasma GDF-15 concentrations increased with rising BMI (Figure 4A). The most pronounced elevations were detected in patients with BMI values exceeding 30 kg/m². In the 30–34.9 kg/m² BMI subgroup, GDF-15 levels showed an increasing trend compared with controls (1450.2 ± 251.0 pg/mL vs. 572.8 ± 82.8 pg/mL), though this difference was not statistically significant ($p = 0.266$). In contrast, patients with extreme obesity (BMI >35 kg/m²) exhibited significantly higher GDF-15 levels than controls ($p < 0.05$) (Figure 4A).

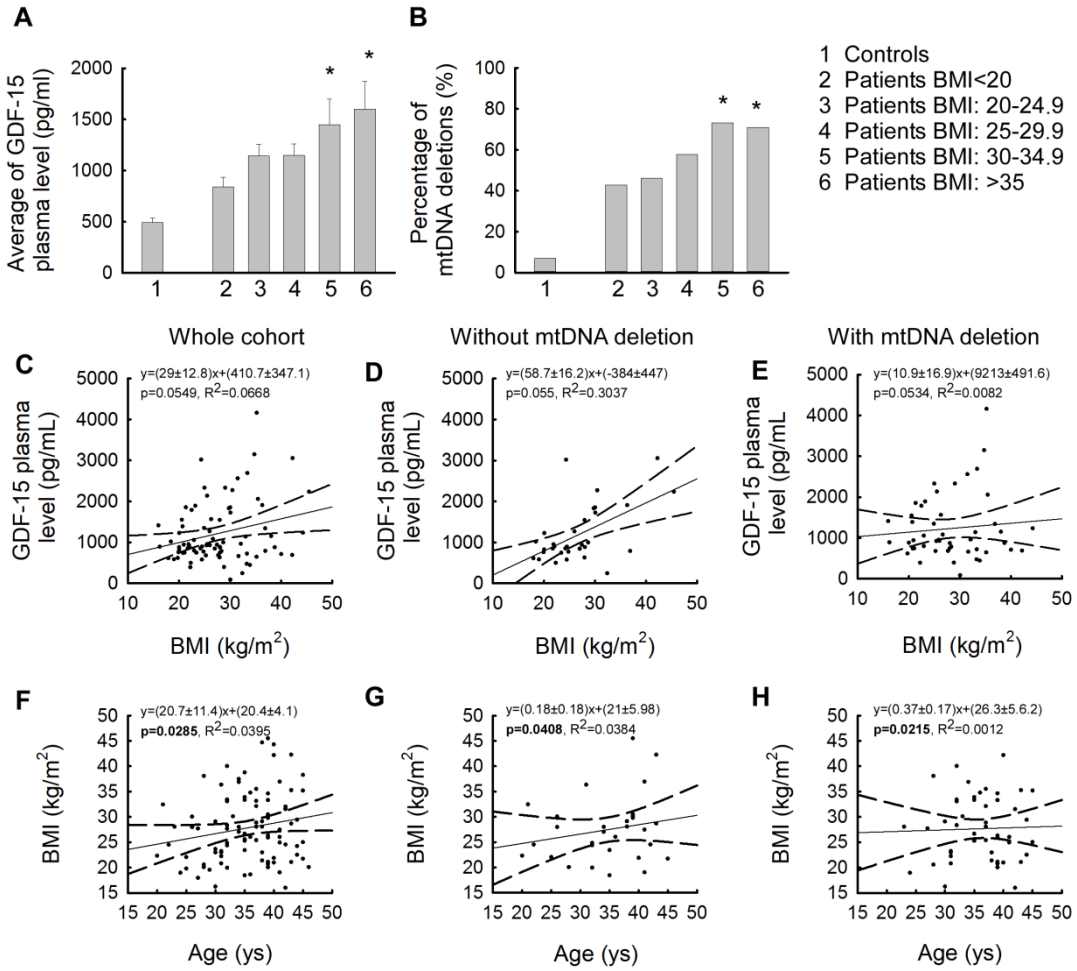

Figure 4. Associations between GDF-15, BMI, age, and mtDNA deletions.

Panels A–H illustrate GDF-15 and mtDNA deletion prevalence across BMI ranges and correlation analyses. (109)

(A) Mean ± SEM plasma GDF-15 levels in different body mass index ranges, compared with levels in healthy controls (* $p < 0.05$, Kruskal–Wallis Dunn test). (B) The percentage of individuals with mtDNA deletions in different BMI ranges in the patient group compared to the percentage in healthy controls (* $p < 0.05$, Kruskal–Wallis Dunn test). BMI subgroups: patients with BMI <20 kg/m² (n = 8); patients with BMI 20–24.9 kg/m² (n = 25); patients with BMI 25–29.9 kg/m² (n = 22); patients with BMI 30–34.9 kg/m² (n = 13); and patients with BMI >35 kg/m² (n = >35 kg/m² (n = 22). (C) Correlation between plasma GDF-15 levels and BMI values in the whole patient group ($R^2 = 0.0668$). (D) The correlation between plasma GDF-15 levels and BMI values in the subgroup of patients without mtDNA deletions ($R^2 = 0.3071$). (E) The correlation between plasma GDF-15 levels and BMI values in the subgroup of patients

with mtDNA deletions (R^2 = 0.00882). (F) The correlation between BMI and age at examination in the whole patient group (R^2 = 0.03395). (G) The correlation between BMI values and age at examination in patients without mtDNA deletions (R^2 = 0.04088). (H) The correlation between BMI values and age at examination in patients with mtDNA deletions ($R^2 = 1.01012 \times 10^{-3}$).

A similar BMI-dependent pattern was observed for mtDNA deletion prevalence. The frequency of mtDNA deletions increased progressively with rising BMI values, exceeding 70% in patients with a BMI greater than 30 kg/m² (Figure 4B). Correlation analysis confirmed a positive association between plasma GDF-15 levels and BMI across the patient cohort (Figure 4C). Interestingly, this association was significantly stronger in patients without mtDNA deletions than in those harbouring single or multiple mtDNA deletions (Figures 4D and 4E). Finally, linear regression analysis of age and BMI revealed a weaker association in patients with mitochondrial dysfunction compared to patients without mtDNA deletions (Figures 4F–H). The relationship between plasma GDF-15 levels and age at the time of examination was analyzed across the entire study population. A comparison of plasma GDF-15 levels in the study and control groups by age was also performed (Figure 5).

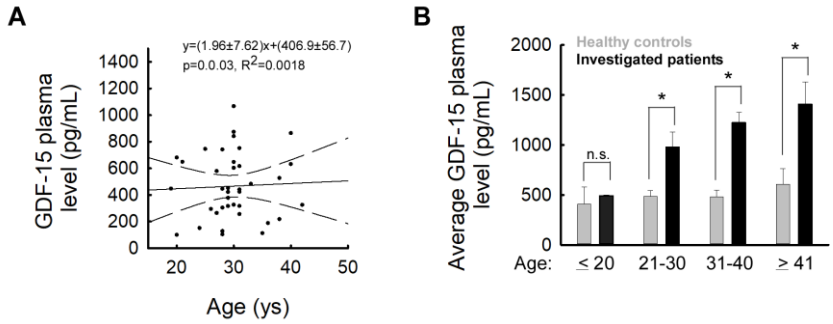

Figure 5. Correlation of plasma GDF-15 levels with age at examination.

(A) Correlation analysis in the total study patient group. (B) Comparison of plasma GDF-15 levels between the study group and healthy controls stratified by age range (109).

4.4. Effect of Insulin-Sensitizing Treatments on Plasma GDF-15 Levels

To evaluate the association between treatments for insulin resistance and plasma GDF-15 levels, the patients were categorized into groups according to their therapeutic

regimen. Of the patients in the study cohort, 32 received only vitamin and dietary supplementation, 26 were treated with metformin, and 23 received a combination of metformin and a glucagon-like peptide-1 (GLP-1) receptor agonist (either liraglutide or semaglutide).

Patients with elevated plasma GDF-15 levels required a significantly higher daily dose of metformin than those with normal levels (1,805.6 ± 634.6 mg/day vs. 1,284.1 ± 786.3 mg/day, $p < 0.05$; Figure 6A). Across all treatment groups, plasma GDF-15 levels were significantly higher than in healthy controls. The lowest GDF-15 concentrations were observed in patients receiving only vitamin and dietary supplementation (982.2 ± 484.2 pg/mL versus 572.8 ± 82.8 pg/mL in controls, $p < 0.05$), whereas the highest levels were detected in patients treated with a combination of metformin and a GLP-1 receptor agonist (Figure 6B).

Linear regression analysis demonstrated a positive correlation between the daily metformin dosage and plasma GDF-15 levels across the entire cohort (Figure 5C). Notably, this relationship was maintained in both patients with and without mtDNA deletions (Figures 6D and 6E). However, when metformin exposure was normalised to body weight (mg/kg/day), the association with GDF-15 became less linear, particularly in patients harbouring mtDNA deletions compared with those without (Figures 6F–H).

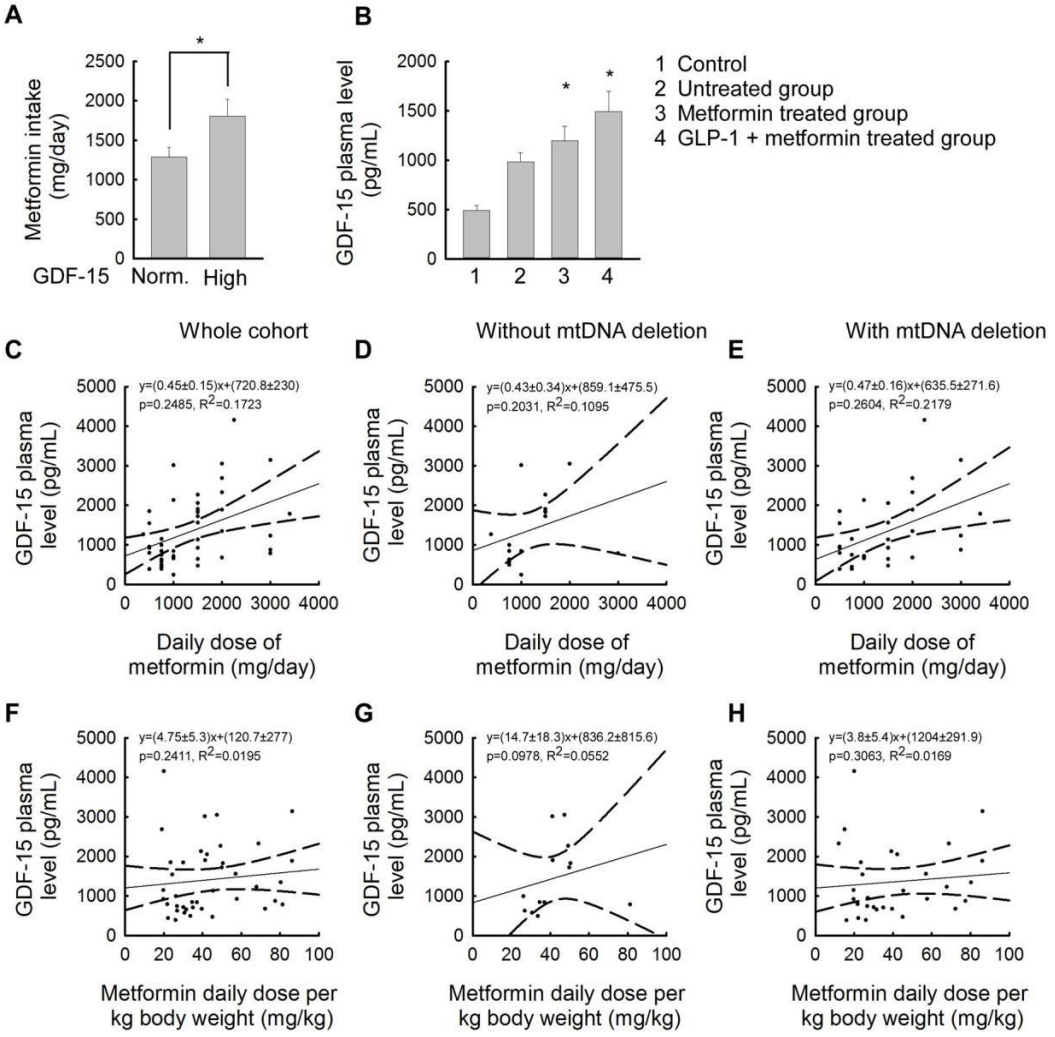

Figure 6. Relationship between plasma GDF-15 and metformin treatment.

Panels A–H show daily dose comparisons and correlation analyses. (109)

(A) Mean daily metformin intake (expressed as mg/day) in subgroups of patients with normal (n = 40) and elevated (n = 9) plasma GDF-15 levels (*: $p < 0.05$, Mann-Whitney U test). (B) Mean plasma GDF-15 levels (expressed as pg/mL) in healthy controls (N = 41) (1); patients treated with vitamins and supplements only (N = 32) (2); patients with metformin-only treatment (N = 26) (3); and patients with combined metformin and GLP-1 receptor agonist treatment (N = 23) (* $p < 0.05$, Kruskal-Wallis Dunn). (C) The correlation between plasma GDF-15 levels (expressed as pg/mL) and mean daily metformin intake (expressed as mg/day) in the entire metformin-treated patient group (R^2: 0.1723) (n = 49). (D) The correlation between plasma GDF-15 levels (expressed as pg/mL) and mean daily metformin intake (expressed as mg/day) in patients without mtDNA deletions (R^2: 0.1019; n = 17). (E) Correlation between plasma GDF-15 levels (expressed as pg/mL) and mean daily metformin intake (expressed as mg/day) in patients with mtDNA deletions (R^2: 0.2172; n = 32). (F) The correlation between plasma GDF-15 levels

(expressed as pg/mL) and the metformin daily dose per kg of body weight (expressed as mg/day) in the entire metformin-treated patient group (R^2: 0.0195) (n = 49). (G) The correlation between plasma GDF-15 levels (expressed as pg/mL) and the metformin daily dose per kg of body weight (expressed as mg/day) in patients without mtDNA deletions (R^2: 0.0553) (n = 17). (H) Correlation between plasma GDF-15 levels (expressed as pg/mL) and metformin dose per kg body weight (expressed as mg/day) in patients with mtDNA deletions (R^2: 0.0169) (n = 32).

4.5. Assessment of Multisystem Symptoms Associated with Mitochondrial Dysfunction and Clinical Characteristics of Patients with Elevated Plasma GDF-15 Levels.

During enrolment, all patients completed a self-administered clinical symptom questionnaire designed to evaluate multisystem involvement. Across the cohort, involvement ranged from 0 to seven organ systems. A significantly greater proportion of mtDNA deletion-positive patients exhibited involvement of more than five organ systems compared with deletion-negative individuals (26% [13/50] vs. 3.2% [1/31]; $\chi^2 = 6.94$, p = 0.01). These findings demonstrate clustering of higher multisystem burden among deletion-positive patients. However, the overall extent of organ system involvement did not differ between patients with elevated and normal plasma GDF-15 levels (Table 1).

Table 1: Distribution of patients by the number of affected organ systems according to mtDNA deletion status and plasma GDF-15 levels (110).

The table presents the proportion of patients with ≤5 or >5 affected organ systems in the whole cohort and stratified by mitochondrial DNA (mtDNA) deletion status (negative vs. positive) and by plasma GDF-15 concentration (normal vs. elevated). Data are shown as percentages with case numbers (n/N). A significant association was found between mtDNA deletion status and multisystemic involvement ($\chi^2 = 6.94$, p = 0.01), while no significant difference was observed between normal and elevated GDF-15 subgroups (p = 1.0).

(Abbreviations: mtDNA, mitochondrial DNA; del., deletion, Neg. – negatív. Pos. – positive).

Number of affected organ systems	Whole cohort	mtDNA del. Neg	mtDNA del. Pos.	Chi2	Fisher's Exact test	Normal GDF-15	Elevated GDF-15	Chi2	Fisher's Exact test
0-5	82.7% (67/81)	96.8% (30/31)	74% (37/50)			82.6% (57/69)	83.3% (10/12)		
				6.94	***0.01***			0.0038	1
>5	17.3% (14/81)	3.2% (1/31)	26% (13/50)			17.4% (12/69)	16.7% (2/12)		

Patients harboring mtDNA deletions demonstrated a higher frequency of muscle-related symptoms, gastrointestinal disturbances, early-life psychomotor developmental delays, psychiatric disorders – predominantly depression – and autoimmune conditions. The latter encompassed Hashimoto's thyroiditis, systemic lupus erythematosus, vitiligo, ankylosing spondylitis, antiphospholipid syndrome, and undifferentiated autoimmune syndromes. These data were collected via patient questionnaires and review of medical records. Among these features, only the prevalence of additional endocrine manifestations reached statistical significance ($\chi^2 = 6.45$, $p < 0.05$) (Table 2). When stratifying patients by plasma GDF-15 levels, no significant differences were observed in the occurrence of organ-specific symptoms. Although autoimmune conditions appeared more common in individuals with elevated GDF-15, this trend did not attain statistical significance (Table 2).

Table 2. Organ-system-specific clinical symptoms stratified by mtDNA deletion status and plasma GDF-15 levels (110)

The percentage of complaints and symptoms relating to specific organ systems in the whole patient cohort (n = 81) (1), the subgroup without mtDNA deletion (n = 31) (2), the subgroup with single or multiple mtDNA deletions (n = 50) (3), the subgroup with normal GDF-15 plasma levels (expressed as pg/mL) (n = 69) (4), and the subgroup with elevated GDF-15 plasma levels (n = 12) is shown below. These figures are based on self-completion questionnaire responses and available medical record data. The significance between pairs of groups was calculated using chi-squared and Fisher's exact tests. Significant differences are marked in bold ($p < 0.05$). (Abbreviations: mtDNA, mitochondrial DNA; del., deletion; NA, not applicable; GI, gastrointestinal; TIA, transient ischaemic attack.)

Clinical symptoms	Whole cohort	mtDNA del. Neg	mtDNA del. Pos	Fisher Exact test	Chi²	Normal GDF-15	Elevated GDF-15	Fisher Exact test	Chi²
Exercise intolerance	40.0% *(32/81)*	29.4% *(9/31)*	42.1% *(21/50)*	0.34	1.38	39.7% *(27/69)*	41.70% *(5/12)*	1	0.03
Visual impairment	8.2% *(7/81)*	8.8% *(3/31)*	7.0% *(4/50)*	0.71	0.08	8.2% *(6/69)*	8.30% *(1/12)*	1	0.01
Hearing impairment	3.5% *(3/81)*	0.0% *(0/31)*	5.3% *(3/50)*	0.28	na	4.1% *(3/69)*	0.00% *(0/12)*	1	na
GI symptoms	43.5% *(35/81)*	32.4% *(10/31)*	45.6% *(23/50)*	0.25	1.49	43.8% *(30/69)*	41.70% *(5/12)*	1	0.01
Cardiac involvement	14.1% *(11/81)*	17.6% *(5/31)*	10.5% *(5/50)*	0.49	0.66	16.4% *(11/69)*	0.00% *(0/12)*	0.21	na
TIA/Stroke	0.0% *(0/81)*	0.0% *(0/31)*	0.0% *(0/50)*	1	na	0.0% *(0/69)*	0,00% *(0/12)*	1	na
Psychomotor delay	9.4% *(8/81)*	2.9% *(1/31)*	12.3% *(6/50)*	0.24	1.86	9.6% *(7/69)*	8.30% *(1/12)*	1	0.04
Neurological symptoms	7.1% *(6/81)*	5.9% *(2/31)*	7.0% *(4/50)*	1	0.05	8.2% *(6/69)*	0.00% *(0/12)*	0.59	na
Psychiatric involvement	35.3% *(29/81)*	26.5% *(8/31)*	36.8% *(18/50)*	0.46	0.91	37.0% *(26/69)*	25.00% *(3/12)*	0.53	0.72
Autoimmune involvement	25.9% *(21/81)*	17.6% *(5/31)*	28.1% *(14/50)*	0.29	1.50	24.7% *(17/69)*	33.30% *(4/12)*	0.5	*0.40*

Heat or cold intolerance	56.5% *(46/81)*	50.0% *(16/31)*	54.4% *(27/50)*	1	0.12	56.2% *(39/69)*	66.6% *(8/12)*	0.75	0.45
Other endocrine symptoms	45.7% *(38/81)*	29% *(9/31)*	57.1% *(29/50)*	**0.013**	**6.45**	46.4% *(32/69)*	41.7% *(5/12)*	1	0.09

Based on a recent meta-analysis (112), age-adjusted cut-off values were applied to define elevated GDF-15 levels: <30 years: 2195 pg/mL; 30–39 years: 1950 pg/mL; 40–49 years: 1804 pg/mL. This identified 12 out of 81 patients with elevated plasma GDF-15. Among these patients, 11 exhibited an elevated BMI, while the remaining individual had a BMI at the upper limit of the normal range (25 kg/m²). Notably, eight of the twelve patients with elevated GDF-15 levels also carried mtDNA deletions, consistent with our previous observations; however, the small sample size limits formal statistical conclusions. In the cohort with normal GDF-15 levels, 45 out of 69 individuals (65.2%) presented with an elevated BMI (>25 kg/m²).

The distribution of symptoms among cases with elevated GDF-15 is shown in Table 4. Among patients with elevated GDF-15 levels, the following symptoms were reported: exercise intolerance (five patients), visual impairment (one patient), and gastrointestinal complaints (five patients). Cardiovascular symptoms were observed in only one patient, whereas another exhibited psychomotor developmental delay during early childhood. Psychiatric symptoms, as well as thyroid or other endocrine disorders, were each present in three patients, while autoimmune disorders were documented in four individuals (33.3%). A total of eight out of twelve patients (66.6%) displayed pronounced intolerance to cold or heat. Notably, none of the patients reported a history of hearing loss, transient ischemic attacks or stroke, or other neurological manifestations (Table 3).

Table 3. Frequency of concurrent symptoms in different organ systems in patients with elevated GDF-15 levels (110).

The frequency of these symptoms in different organ systems was based on responses to a self-completion questionnaire and available medical record data. (Abbreviations: P1–12, Patient 1–12).

Clinical Symptoms	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
Exercise intolerance (n = 5)	+	–	+	+	–	–	–	–	–	+	+	–
Visual impairment (n = 1)	–	+	–	–	–	–	–	–	–	–	–	–
Hearing impairment (n = 0)	–	–	–	–	–	–	–	–	–	–	–	–
GI symptoms (n = 5)	+	–	–	+	+	+	+	–	–	–	–	–
Cardiac involvement (n = 1)	–	–	–	–	–	–	–	+	–	–	–	–
TIA/Stroke (n = 0)	–	–	–	–	–	–	–	–	–	–	–	–
Psychomotor delay (n = 1)	+	–	–	–	–	–	–	–	–	–	–	–
Neurological symptoms (n = 0)	–	–	–	–	–	–	–	–	–	–	–	–
Psychiatric involvement (n = 3)	+	–	–	+	+	–	–	–	–	–	–	–
Autoimmune involvement (n = 4)	+	–	–	+	–	+	+	–	–	–	–	–
Heat or cold intolerance (n = 8)	+	+	+	+	+	+	–	–	–	+	–	+
Other endocrine symptoms (n = 5)	–	–	–	+	+	+	+	–	–	–	+	–

4.6. Distribution of Clinical Manifestations Across Distinct Patient Subgroups

Given that mitochondrial dysfunction generally impacts multiple organ systems and presents with a broad spectrum of clinical symptoms, we examined the distribution of manifestations across different clinical subgroups. No significant difference was observed in the number of affected organ systems between subgroups. Analysis of symptom patterns within each subgroup indicated that visual impairment, cardiovascular involvement, and neurological problems – symptoms typically associated with aging – were most prevalent in the IR-PCOS group. In contrast, these manifestations were absent in the IR-POI patients (Table 4 and Figure 7).

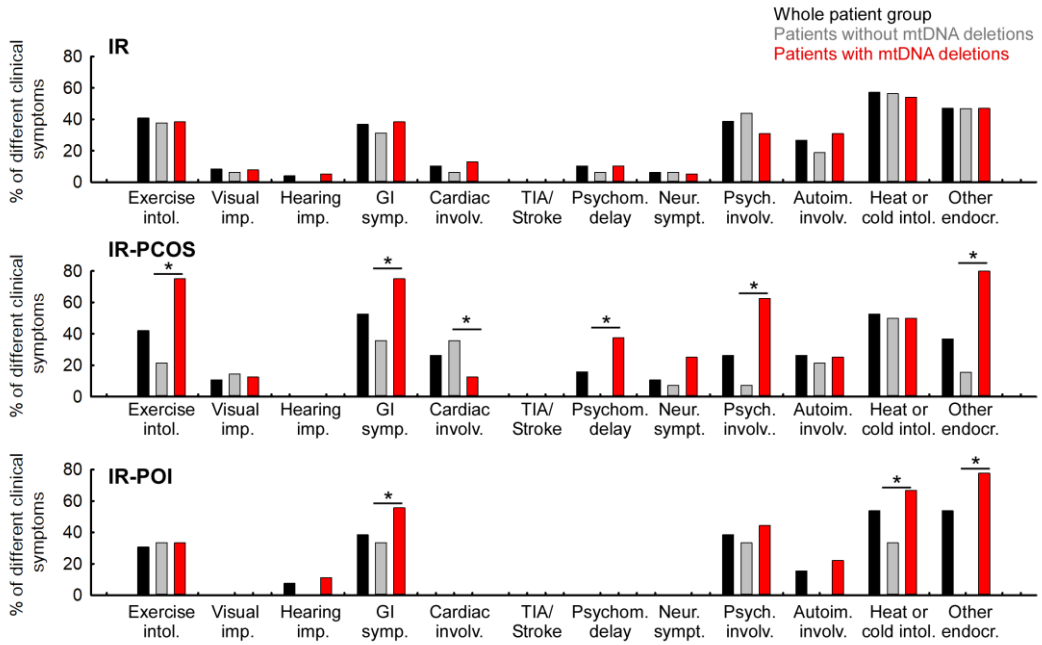

Figure 7. Distribution of mtDNA deletions and clinical subgroups. - Organ-system involvement by subgroup and mtDNA deletion status (110)

The distribution of mtDNA deletions (single or multiple) in the entire patient group and in each patient subgroup is shown, as is the percentage prevalence of each organ system symptom in the IR-only, IR-PCOS, and IR-POI subgroups. (A) Distribution of individuals with and without single or multiple mtDNA deletions in: the whole patient group (1); the IR-only subgroup (n = 49; $n_{del.\ neg}$ = 15, $n_{del.\ pos}$ = 34) (2); the IR-PCOS subgroup (n = 19; $n_{del.\ neg}$ = 13, $n_{del.\ pos}$ = 6) (3); the IR-POI subgroup (n = 13; $n_{del.\ neg}$ = 4, $n_{del.\ pos}$ = 9). The significance between pairs of groups was calculated using the chi-square test (*: $p < 0.05$). (*: $p < 0.05$). (B) Prevalence of organ system symptoms by mtDNA deletion status of patients, considering the group or subgroup to be 100%. Percentage of organ system involvement in the whole patient group (black columns), patients without mtDNA deletions (grey columns), and patients with mtDNA deletions (red columns). The Mann-Whitney U test was used to determine significance between groups (*: $p < 0.05$). (Abbreviation: Exercise intolerance (Exercise intol.); visual impairment (Visual imp.); hearing impairment (Hearing imp.); gastrointestinal symptoms (GI symp.); cardiovascular involvement (Cardiac involv.); early childhood psychomotor developmental delay (Psychom. delay); neurological symptoms (Neurological sympt.); psychiatric involvement (Psych. involv.); autoimmune involvement (Autoim. involv.); heat or cold intolerance (Heat or cold intol.); other endocrine symptoms (Other endocr.).

Next, we examined the prevalence of organ-specific symptoms in the IR-only, IR-PCOS, and IR-POI subgroups, and their association with mtDNA deletion. Percentages were

calculated relative to the size of each subgroup. Physical exercise intolerance was similarly frequent in the IR-only and IR-PCOS groups (40.8% vs. 42.1%), but less common in the IR-POI group (30.8%) (Table 3). Interestingly, 75% of IR-PCOS patients with mtDNA deletions reported this symptom, whereas deletion status had no impact in the IR-only or IR-POI subgroups (Figure 7).

Visual impairment affected 8.2% of IR-only and 10.5% of IR-PCOS patients, with no cases in the IR-POI group, and showed no association with mtDNA deletion status (Table 4, Figure 1B). Gastrointestinal problems were most frequent in the IR-PCOS group, particularly among patients harboring mtDNA deletions (Table 2, Figure 1B). Cerebrovascular events, including transient ischemic attacks and stroke, were absent in all subgroups (Table 4, Figure 5).

Cardiovascular symptoms, delayed psychomotor development in early childhood, and neurological symptoms were absent in the IR-POI group, but were most prevalent in the IR-PCOS group (Table 4 and Figure 7). Within the IR-PCOS subgroup, delayed psychomotor development and neurological involvement showed a strong correlation with the presence of mtDNA deletions (Figure 7). Psychiatric symptoms were primarily observed in the IR-only and IR-POI groups, where mtDNA deletion status did not appear to influence their occurrence (Figure 6). In contrast, despite the low overall prevalence of psychiatric symptoms in the IR-PCOS group, a significant association with mtDNA deletions was detected (Figure 7).

Autoimmune manifestations were least frequent in the IR-POI group and were observed exclusively in patients harboring mtDNA deletions (Tables 2 and 4). The prevalence of heat and cold intolerance was comparable across all subgroups (Table 4), although these symptoms were more common among IR-POI patients with mtDNA deletions (Figure 1B). Furthermore, other endocrine abnormalities occurred significantly more frequently in individuals with mtDNA deletions within both the IR-PCOS and IR-POI subgroups (Figure 1B).

Table 4. Organ-system symptoms in IR, IR-PCOS, and IR-POI subgroups (110)

Percentage of specific organ system complaints and symptoms across subgroups. Chi-square and Fisher's exact test were used for significance. In statistical analyses, both the IR-PCOS and IR-POI were compared to the IR-only. NA, not applicable.

Clinical symptoms	IR	IR-PCOS	Fisher Exact test	Chi2	IR-POI	Fisher Exact test	Chi2
		IR vs. IR-PCOS			IR vs. IR-POI		
Exercise intolerance	40.8% *(20/49)*	42.1% *(8/19)*	1	0.01	30.8% *(4/13)*	0.44	0.75
Visual impairment	8.2% *(4/49)*	10.5% *(2/19)*	0.67	0.10	0.0% *(0/13)*	na	0.57
Hearing impairment	4.1% *(2/49)*	0.0% *(0/19)*	1	na	7.7% *(1/13)*	0.29	0.51
GI symptoms	36.7% *(18/49)*	52.6% *(10/19)*	0.28	1.43	38.5% *(5/13)*	0.01	1
Cardiac involvement	10.2% *(5/49)*	26.3% *(5/19)*	0.13	2.83	0.0% *(0/13)*	na	0.57
TIA/Stroke	0.0% *(0/49)*	0.0% *(0/19)*	1	na	0.0% *(0/13)*	na	1
Psychomotor delay	10.2% *(5/49)*	15.8% *(3/19)*	0.68	0.41	0.0% *(0/13)*	na	0.57
Neurological symptoms	6.1% *(3/49)*	10.5% *(2/19)*	0.61	1,03	0.0% *(0/13)*	na	1
Psychiatric involvement	38.8% *(19/49)*	26.3% *(5/19)*	0.41	0.93	38.5% *(5/13)*	0.00	1

Autoimmune involvement	26.5% *(13/49)*	26.3% *(5/19)*	1	0.01	15.4% *(2/13)*	0.70	0.49
Heat or cold intolerance	57.1% *(28/49)*	52.6% *(10/19)*	0.79	0.11	53.8% *(7/13)*	0.05	1
Other endocrine symptoms	46.9% *(23/49)*	36.8% *(7/19)*	0.23	0.81	36.8% *(7/13)*	0.66	0.79

4.7. Hormonal Parameters and Their Association with Plasma GDF-15

The key baseline metabolic and endocrine parameters investigated in the study are summarized in Table 5.

Table 5: The most important endocrine parameters and hormonal levels in the 3 subgroups within the whole patient cohort.

The IR-only subgroup (first column); the IR-PCOS subgroup (second column); and the IR-POI subgroup (third column). (*: p<0,05 (IR only vs IR-PCOS); +: p<0,05 (IR only vs IR-POI); #: p<0,05 (IR-PCOS vs IR-POI)) (110).

	IR only	IR-PCOS	IR-POI
Age at examination	37±0.8	30.6±1.2	37.7±1.2
BMI (kg/m2)	28.9±0.9	25.4±1.5	28.2±1.9
AMH (ng/ml)	3±0.5	7.1±1	0.6±0.1
Glucose 0' (mmol/L)	5.3±0.2	5.1±0.2	5.1±0.2
Insulin 0' (µU/mL)	14.3±1.1	11.4±2.2	10.7±1.8
HOMA index	3.4±0.4	2.7±0.6	2.5±0.5
Vitamin D3 (ng/mL)	38.1±2.1	36.1±2.1	41.1±2.5
Glucose 0' (mmol/L)	5.3±0.2	5.1±0.2	5.1±0.2
Glucose 60' (mmol/L)	7.3±0.3	7±0.5	7±0.7
Glucose 90' (mmol/L)	5.6±0.3	4.7±0.1	4.4±0.2
Glucose 120' (mmol/L)	6±0.3	5.2±0.3	6±0.7
Insulin 0' (µU/mL)	14.3±1.1	11.4±2.2	10.7±1.8
Insulin 60' (µU/mL)	82.2±7.6	70.8±13.4	75.5±14
Insulin 90' (µU/mL)	38.1±2.6	25.3±3.2	26.5±2
Insulin 120' (µU/mL)	56.9±6	44.5±9.8	60±14.3
TSH (mlU/L)	2.2±0.2	2.3±0.4	2.8±0.2
T4 (ng/dL)	14.8±0.3	14.1±0.6	14.5±0.7
T3 (ng/dL)	4.9±0.2	4.5±0.3	5.1±0.3
FSH (mlU/mL)	8.5±0.7	6.1±0.3	7.5±0.9
LH (mlU/mL)	5.2±0.5	5.8±0.9	3.3±0.5
Prolactin (mlU/L)	13±0.8	13.9±2.4	12.3±1.7
Estradiol (mlU/mL)	70.2±10.1	87.8±23.8	69.7±10.3
Progesterone (nmol/L)	0.7±0.2	3.1±1	0.2±0.1
Total testosterone (nmol/L)	0.4±0.1	0.5±0.1	0.5±0.1
Free testosterone (nmol/L)	4.6±0.2	4.6±1.3	1.5±0.5
SHBG (nmol/L)	53.2±2.1	59.1±6.9	65.3±8.3

The relationship between plasma GDF-15 concentrations and hormones relevant to female reproductive function was analysed with consideration of mtDNA deletion status. Figure 8 summarises mean levels of thyroid, metabolic, and reproductive hormones in the total cohort and in subgroups stratified according to mtDNA deletion status and plasma GDF-15 levels. Outliers exceeding two standard deviations were excluded, and between-group comparisons were assessed using analysis of variance.

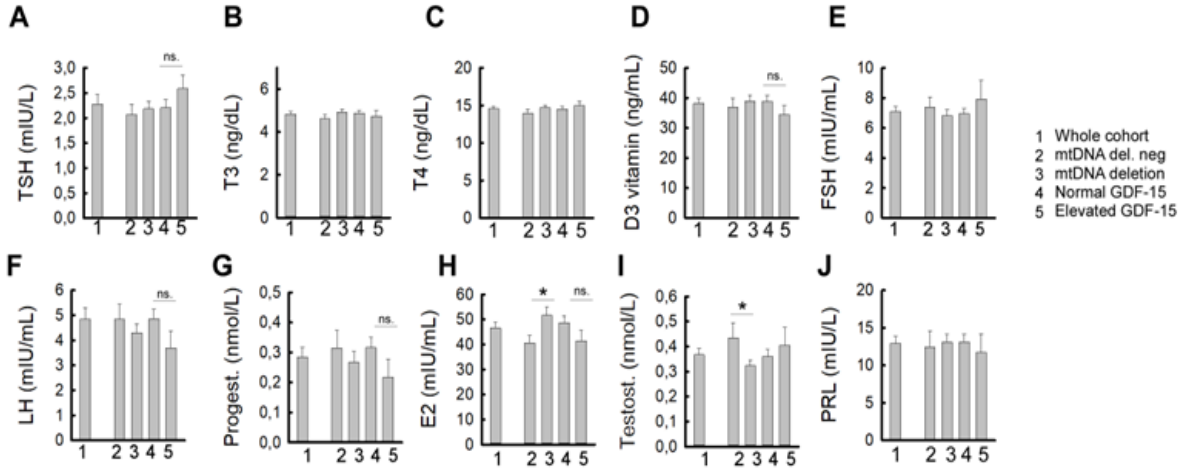

Figure 8. Hormonal parameters in relation to mtDNA deletion status and GDF-15 levels (110).
(A) TSH, (B) T3, (C) T4, (D) vitamin D3, (E) FSH, (F) LH, (G) progesterone, (H) oestradiol, (I) testosterone and (J) prolactin are shown for the whole cohort (1) and the following subgroups: without mtDNA deletions (2); with mtDNA deletions (3); with normal plasma GDF-15 levels (4); and with elevated GDF-15 levels (5). * = $p < 0.05$, ns = not significant.

Based on the underlying clinical phenotypes, differences in anti-Müllerian hormone (AMH), progesterone, and fasting insulin levels were anticipated. As expected, progesterone levels differed significantly between the IR-PCOS and IR-POI subgroups, while fasting insulin levels were significantly higher in the IR-only subgroup compared with the IR-PCOS and IR-POI groups. No other hormonal parameters showed significant differences between the clinical subgroups. Notably, the IR-PCOS subgroup was significantly younger than the other groups. To assess associations between hormone levels and plasma GDF-15 concentrations, simple and multiple linear regression analyses were performed, with age and body mass index (BMI) included as covariates. Analyses were performed in the total cohort as well as in subgroups stratified by mtDNA deletion

status. Among thyroid parameters, only thyroxine (T4) exhibited an association with plasma GDF-15 levels. In the overall cohort, multiple linear regression identified T4 as an independent predictor of GDF-15 ($\beta = 88.4$, $p = 0.035$), whereas age and BMI did not emerge as significant covariates. Although the overall model reached statistical significance ($R^2 = 0.139$, $p = 0.043$), assumptions of normality and homoscedasticity were partially violated (Figure 9, Table 6).

Stratified analyses revealed divergent patterns according to mtDNA deletion status. In deletion-negative patients, the regression model was significant ($R^2 = 0.434$, $p = 0.015$), with BMI emerging as the only significant predictor of plasma GDF-15 levels, while T4 showed a non-significant trend. In contrast, no significant univariate association between T4 and GDF-15 was observed in deletion-positive patients. Upon incorporating age and BMI into the multivariate analysis, T4 demonstrated nominal significance, although the model as a whole failed to achieve statistical significance. Notably, the regression slope for T4 was more than two-fold steeper in deletion-positive patients than in deletion-negative individuals, suggesting a stronger – but exploratory – association in the presence of mitochondrial dysfunction (Figure 9).

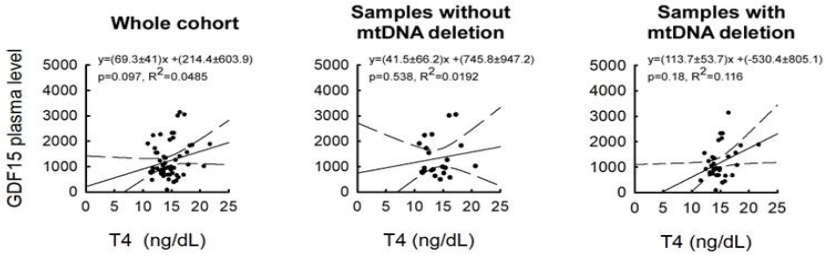

Figure 9. Linear regression analysis of plasma GDF-15 concentrations in relation to circulating T4 levels. Associations are shown for the total patient cohort, for patients without detectable mitochondrial DNA deletions, and for patients with single or multiple mtDNA deletions. Solid lines represent fitted linear regression models, while dashed lines indicate the corresponding 95% confidence intervals (110).

With respect to reproductive hormones, significant subgroup differences were observed only for estradiol and testosterone. Patients with mtDNA deletions exhibited higher estradiol and lower total testosterone levels compared with deletion-negative individuals. No additional reproductive hormones showed significant differences between subgroups, and after controlling for age and BMI, univariate and multivariate regression analyses did

not identify any significant associations between GDF-15 and reproductive hormone levels.

Finally, in deletion-negative individuals, a multivariate model incorporating age, BMI, and vitamin D_3 levels significantly predicted plasma GDF-15 concentrations ($R^2 = 0.353$, $p = 0.037$). Although none of the individual predictors reached statistical significance, vitamin D3 consistently showed a negative regression coefficient, suggesting a potential inverse relationship with GDF-15 levels in this subgroup (Table 6).

Table 6. Results of multiple linear regression analyses assessing the association between plasma GDF-15 levels and selected hormonal parameters (thyroxine [T4], vitamin D3, and total testosterone), adjusted for age and body mass index, in the total cohort and in subgroups stratified by mtDNA deletion status (110).

Reported values represent unstandardized coefficients (b) along with their standard errors (SE), t-statistics (t), and two-tailed p-values for each predictor. 'Model p' indicates the p-value from the omnibus F-test, while 'Model R^2' corresponds to the coefficient of determination. Subgroups are defined as **Del-neg** for mtDNA deletion-negative and **Del-pos** for mtDNA deletion-positive. N refers to the number of cases with complete data included in the respective model. Statistical significance was considered at $p < 0.05$.

Predictor	Subgroup	b (coeff.)	SE	t	p-value	Model p	Model R2
T4	Total (N=58)	88.39	40.832	2.165	0.035	0.043	0.139
T4	mtDNA del. neg. (N=22)	99.565	55.612	1.79	0.09	0.015	0.434
T4	mtDNA del. pos. (N=36)	114.886	55.877	2.056	0.048	0.196	0.056
Vitamin D3	mtDNA del. neg. (N=22)	-10.187	10.488	-0.971	0.344	0.035	0.402
Testosterone	mtDNA del. pos. (N=20)	3567.303	1281.813	2.783	0.013	0.016	0.096

4.8. Ovarian Reserve Markers, Age, and Mitochondrial Dysfunction

Subsequently, the relationship between ovarian reserve markers and mtDNA deletion status, as well as plasma GDF-15 concentrations, was analysed (Figure 10). Anti-Mullerian hormone (AMH) is commonly employed as a marker of ovarian reserve, often alongside the AMH/FSH ratio (Figure 10). To explore potential associations with mitochondrial stress, we analyzed correlations between plasma GDF-15 levels and both AMH concentrations and the AMH/FSH ratio. These analyses were confined to the insulin resistance (IR)-only subgroup and were further stratified according to mtDNA deletion status. Patients in the IR-polycystic ovary syndrome (PCOS) and IR-primary ovarian insufficiency (POI) subgroups were excluded due to their unique hormonal characteristics and underlying pathophysiology.

While mean AMH levels tended to be lower in participants with elevated GDF-15, this difference did not achieve statistical significance (Figure 10A). Conversely, among mtDNA deletion-positive individuals, the AMH/FSH ratio was significantly lower in those with elevated GDF-15 compared to participants whose GDF-15 remained within the normal range ($p < 0.05$; Figure 10C), suggesting a potential link between mitochondrial dysfunction and reduced ovarian reserve.

Within the studied cohort ($n = 35$), AMH demonstrated a strong negative correlation with age ($R^2 = 0.501$, $p < 0.001$, $\beta = -2.18$), reaffirming its role as an indicator of reproductive aging. This inverse relationship persisted when analyses were stratified by mtDNA deletion status: both deletion-negative ($R^2 = 0.635$, $p = 0.006$; $\beta = -1.93$) and deletion-positive ($R^2 = 0.486$, $p < 0.001$; $\beta = -2.25$) subgroups exhibited significant negative associations, with the deletion-positive group showing a steeper decline, indicative of a more rapid age-related reduction in AMH levels.

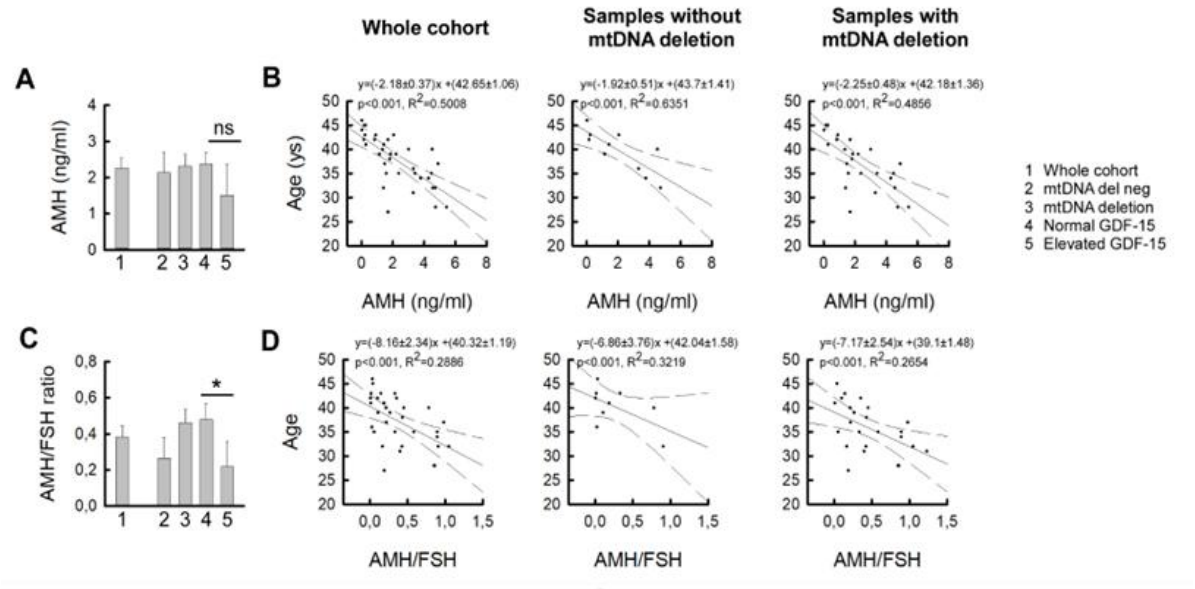

Figure 9. Relationships among ovarian reserve markers, age, mtDNA deletion status, and GDF-15 levels (110).

(A) Serum anti-Müllerian hormone (AMH) concentrations and (C) AMH/FSH ratios are shown for the following subgroups: (1) the entire cohort, (2) mtDNA deletion-negative, (3) mtDNA deletion-positive, (4) normal GDF-15, and (5) elevated GDF-15. Data are expressed as mean ± SEM. Statistical comparisons were conducted using one-way ANOVA followed by post hoc analyses. * indicates $p < 0.05$; ns denotes non-significant differences. correlation analyses of age with AMH (B) and the AMH/FSH ratio (D) for the full cohort (left), deletion-negative samples (middle), and deletion-positive samples (right). Linear regression lines with 95% confidence intervals are illustrated, with solid lines representing the regression and dashed lines the 95% confidence interval. Corresponding regression equations, coefficients of determination (R^2), and p-values are provided within each panel.

Similarly, in the patient cohort under study (n = 32), the AMH/FSH ratio demonstrated a significant negative correlation with age ($R^2 = 0.289$, $p = 0.002$, $\beta = -8.16$). This inverse association remained statistically significant within the deletion-positive subgroup ($R^2 = 0.265$, $p = 0.010$, $\beta = -7.17$). A similar trend was observed in the deletion-negative subgroup ($\beta = -6.86$), although it did not reach statistical significance ($R^2 = 0.322$, $p = 0.111$; power = 0.351). Notably, both the slope and intercept were lower in the deletion-positive group, suggesting that equivalent AMH/FSH ratios occurred at younger ages and

declined more rapidly over time, consistent with accelerated reproductive aging in the context of mitochondrial dysfunction.

Among patients without mtDNA deletions (n = 23), a multiple regression model including age, BMI, and vitamin D3 levels significantly predicted plasma GDF-15 concentrations ($R^2 = 0.353$, $p = 0.037$). Although none of the individual predictors reached statistical significance on their own, their combined influence accounted for a substantial portion of the observed variance. Of particular interest, the relationship with vitamin D3 remained negative ($\beta = -16.28$, $p = 0.368$), suggesting that lower vitamin D3 levels may contribute to higher GDF-15 concentrations in this subgroup (Table 6).

5. DISCUSSION

Mitochondrial dysfunction has been increasingly recognized as a contributing factor in a wide range of metabolic, endocrine, and reproductive disorders (113–115). In particular, insulin resistance (IR), polycystic ovary syndrome (PCOS), and premature ovarian insufficiency (POI) represent overlapping clinical phenotypes in which mitochondrial impairment may exacerbate systemic metabolic disturbances and reproductive aging. However, reliable, non-invasive biomarkers that reflect mitochondrial stress in these conditions remain limited.

In this dissertation, we examined the role of two potential biomarkers – plasma growth differentiation factor 15 (GDF-15) and mitochondrial DNA (mtDNA) deletions – in characterizing mitochondrial stress in insulin-resistant women with or without reproductive endocrine dysfunction. GDF-15 is a stress-responsive cytokine implicated in energy metabolism and immune regulation, while mtDNA deletions represent structural genomic damage within mitochondria. By assessing both markers simultaneously, this work aimed to provide a more comprehensive characterization of mitochondrial involvement in IR-related endocrine disorders. Our findings support the idea that GDF-15 and mtDNA deletions capture different aspects of mitochondrial dysfunction. GDF-15 reflects an inducible, systemic stress response, whereas mtDNA deletions represent DNA-based markers of cumulative mitochondrial genome damage.

Our results demonstrated significantly elevated plasma GDF-15 levels in IR and IR-PCOS patients compared with healthy controls, with the highest concentrations observed in the IR-only subgroup. In parallel, mtDNA deletions – predominantly multiple deletions – were more prevalent in patients than in controls, again most frequently in the IR-only group. Although no direct correlation was observed between GDF-15 levels and mtDNA deletion status, both markers were associated with a higher BMI and advancing age, supporting their relevance to metabolic burden and processes related to biological aging.

GDF-15 levels showed statistically significant associations with insulin concentrations at 60 and 120 minutes during oral glucose tolerance testing, higher HOMA-IR indices, and greater metformin exposure. Stratification by BMI revealed progressively increasing GDF-15 levels across BMI categories, with the highest values observed in individuals

with severe obesity (BMI >35 kg/m²). A similar pattern was observed for mtDNA deletions. Together, these findings support the involvement of mitochondrial stress in metabolic inflexibility and disease severity in insulin-resistant women.

Analysis of multiorgan symptom burden demonstrated a descriptively higher prevalence of muscular, gastrointestinal, neurological, psychiatric, and endocrine symptoms in mtDNA deletion-positive patients. Although statistical significance was reached primarily for endocrine manifestations, the overall distribution pattern appears broadly consistent with the multisystemic presentation described in primary mitochondrial disorders (116, 117). In subgroup analyses, IR-PCOS patients with mtDNA deletions exhibited clustering of neurological and gastrointestinal symptoms. Given the limited subgroup size, this exploratory observation should be interpreted cautiously and does not establish a causal inference.

Autoimmune comorbidities were numerically more frequent among patients with elevated GDF-15 levels; however, this difference did not reach statistical significance across subgroup analyses. Therefore, no firm conclusions can be drawn regarding a potential association. Thermoregulatory symptoms, such as heat or cold intolerance, were also more common in GDF-15-positive individuals, suggesting possible autonomic or hypothalamic involvement.

Hormonal profiling revealed significant associations between GDF-15 and several endocrine parameters. Total thyroxine (T4) levels were positively correlated with GDF-15, particularly in mtDNA deletion-positive patients, and multivariate analysis identified T4 as an independent predictor of GDF-15. This finding suggests that mitochondrial dysfunction may amplify the systemic effects of thyroid hormones, potentially through altered tissue-level sensitivity or mitochondrial oxidative capacity (118, 119).

Associations were also observed between mitochondrial markers and sex steroid hormones. Estradiol levels were higher, while total testosterone levels were lower in mtDNA deletion-positive individuals. As mitochondria play a central role in steroidogenesis, impaired mitochondrial function may disrupt hormone synthesis through oxidative stress and enzymatic dysfunction (120, 121). Reduced testosterone levels may

further compromise mitochondrial function, potentially contributing to a self-reinforcing cycle of metabolic and reproductive aging.

In the following sections, we discuss the clinical and pathophysiological implications of these findings, organized around four key domains: (1) GDF-15 and mtDNA deletions as complementary biomarkers of mitochondrial dysfunction, (2) associations with metabolic parameters and insulin resistance, (3) multisystemic manifestations and immune involvement, and (4) hormonal dysregulation and reproductive aging.

5.1. GDF-15 and mtDNA deletions as complementary biomarkers of mitochondrial dysfunction

This study explored the utility of plasma growth differentiation factor 15 (GDF-15) and mitochondrial DNA (mtDNA) deletions as biomarkers of mitochondrial dysfunction in insulin-resistant (IR) women with and without reproductive endocrine disorders. GDF-15, a cytokine responsive to cellular stress, was significantly elevated in IR and IR-PCOS patients compared to healthy controls, aligning with previous studies linking GDF-15 to a broad range of pathological conditions, including cardiovascular, metabolic, inflammatory, and degenerative diseases (122, 123). Likewise, the presence of mtDNA deletions – particularly multiple deletions – was frequent in our cohort, consistent with literature describing their accumulation in metabolic and age-related disorders (124, 125).

Importantly, no significant correlation was observed between mtDNA deletions and GDF-15 levels, suggesting they reflect distinct but potentially complementary dimensions of mitochondrial dysfunction: genomic instability and functional stress response. However, both biomarkers were associated with age and BMI, implying that mitochondrial impairment contributes to systemic aging and metabolic imbalance.

5.2. Mitochondrial alterations are associated with adverse metabolic parameters and increased insulin resistance

Elevated GDF-15 levels were significantly associated with higher HOMA-IR and insulin levels during oral glucose tolerance testing, and nearly all GDF-15-positive patients had a BMI >25 kg/m². Pharmacological treatment profiles revealed that patients on higher doses of metformin – alone or combined with GLP-1 receptor agonists – tended to have higher GDF-15 levels. This may reflect both greater disease severity and metformin's known role as a GDF-15 secretagogue, primarily through intestinal mechanisms (99, 100).

Interestingly, the correlation between GDF-15 levels and metformin dosage was more robust in patients without mtDNA deletions, suggesting that preserved mitochondrial signaling may be necessary for full GDF-15 responsiveness. In obese individuals (BMI >30 kg/m²), both GDF-15 and mtDNA deletions were elevated, consistent with a model in which obesity-related oxidative stress contributes to mitochondrial damage and GDF-15 upregulation.

Despite the anorexigenic effects of GDF-15 via the GFRAL receptor having been demonstrated primarily in experimental and animal models, this effect may be reduced in humans with chronic metabolic disease (96, 126). Possible explanations include impaired receptor function, tissue-level resistance, or altered central signaling due to mitochondrial dysfunction.

5.3. Multisystemic clinical manifestations reflect systemic involvement and immune dysregulation

Patients with mtDNA deletions showed a higher prevalence of systemic symptoms, including muscular, gastrointestinal, neurological, psychiatric, and endocrine disturbances. Notably, a significantly greater proportion of deletion-positive individuals exhibited involvement of more than five organ systems compared with deletion-negative patients (26% vs. 3.2%), which is consistent with clustering of high multisystem burden. (127, 128). In our study, IR-PCOS patients with mtDNA deletions had higher rates of

gastrointestinal symptoms, early-onset psychomotor delay, and neurological findings such as tremor and myoclonus. Given the limited subgroup size, these observations should be interpreted cautiously and regarded as exploratory rather than mechanistic evidence.

Psychiatric symptoms, particularly in the IR-PCOS subgroup, were also more frequent in deletion-positive individuals. The clustering of symptoms within this phenotype supports the hypothesis that mitochondrial dysfunction contributes to the clinical heterogeneity of PCOS by modulating central nervous system, immune, and endocrine pathways (110).

Autoimmune conditions were numerically more frequent among patients with elevated GDF-15; however, this difference did not reach statistical significance. As a TGF-β superfamily member, GDF-15 suppresses lymphocyte proliferation and proinflammatory cytokine production, possibly reflecting a feedback mechanism to counterbalance chronic inflammation (73, 129). Additionally, 66.6% of GDF-15-positive patients reported thermoregulatory symptoms (e.g., heat or cold intolerance), suggestive of autonomic or hypothalamic involvement (110).

5.4. Hormonal dysregulation contributes to impaired reproductive function and accelerated reproductive aging

We observed strong associations between GDF-15 levels and key reproductive and metabolic hormones. Total T4 was significantly correlated with GDF-15, particularly in mtDNA deletion-positive patients. In the total cohort, multivariate analysis identified T4 as an independent predictor of GDF-15.
Although the regression slope appeared steeper in deletion-positive individuals, the subgroup model did not reach overall statistical significance. This suggests that mitochondrial dysfunction may amplify the systemic impact of thyroid hormones, potentially by altering T4-to-T3 conversion or modulating tissue-level sensitivity (121, 130).

Vitamin D3 levels showed no significant difference between groups overall; however, subgroup analysis revealed opposing associations with GDF-15: a negative correlation in

deletion-negative and a non-significant positive trend ($p > 0.05$) was observed in deletion-positive patients. This divergence may reflect compensatory responses to mitochondrial stress, either through supplementation or increased endogenous production (131, 132).

Estradiol levels were higher and total testosterone levels lower in patients with mtDNA deletions. Mitochondria are essential for steroidogenesis, and impaired mitochondrial function can disrupt hormone synthesis via oxidative damage and enzymatic failure (120, 121). Notably, testosterone deficiency may further impair mitochondrial function, creating a vicious cycle that accelerates aging and metabolic decline.

Finally, ovarian reserve markers – particularly the AMH/FSH ratio – were significantly lower in deletion-positive, GDF-15-high individuals (110). Although this subgroup analysis was exploratory and limited by sample size, the consistent direction of association supports a potential link between mitochondrial dysfunction and accelerated reproductive aging. While AMH alone did not reach statistical significance in the subgroup comparison, the reduced ratio suggests compromised follicular activity. These findings are consistent with previous studies linking mitochondrial alterations to reduced ovarian reserve (117, 133, 134). Even after excluding PCOS and POI cases to avoid confounding, the IR-only subgroup showed similar trends, supporting the notion that mitochondrial dysfunction contributes to early reproductive aging independent of overt endocrine pathology. The steeper age-related decline in ovarian reserve markers observed in women with mtDNA deletions supports the concept of accelerated reproductive aging associated with mitochondrial genome instability. Notably, this pattern was evident even in insulin-resistant women without overt PCOS or POI, suggesting that mitochondrial dysfunction may precede the manifestation of reproductive endocrine disorders. Taken together, these findings suggest that mitochondrial DNA instability is linked to a steeper decline in ovarian reserve markers with age. This supports the idea of accelerated reproductive aging in insulin-resistant women with mitochondrial dysfunction.

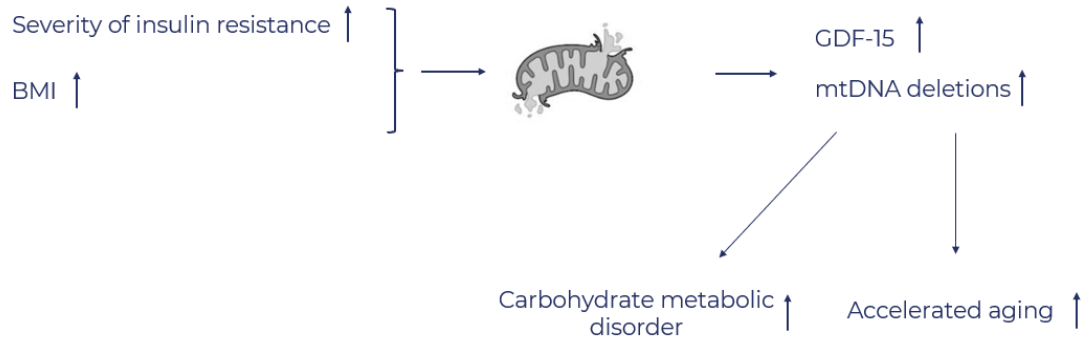

Figure 10. Conceptual model of the relationship between insulin resistance, mitochondrial dysfunction, and reproductive aging (109)

Schematic representation of the proposed relationship between increasing body mass index (BMI) and severity of insulin resistance and their association with mitochondrial dysfunction. Worsening metabolic status is hypothesized to contribute to mitochondrial alterations, reflected by elevated GDF-15 levels and increased mitochondrial DNA (mtDNA) deletions. These mitochondrial changes may, in turn, promote carbohydrate metabolism disorders and contribute to accelerated aging. Arrows indicate the direction of the proposed associations.

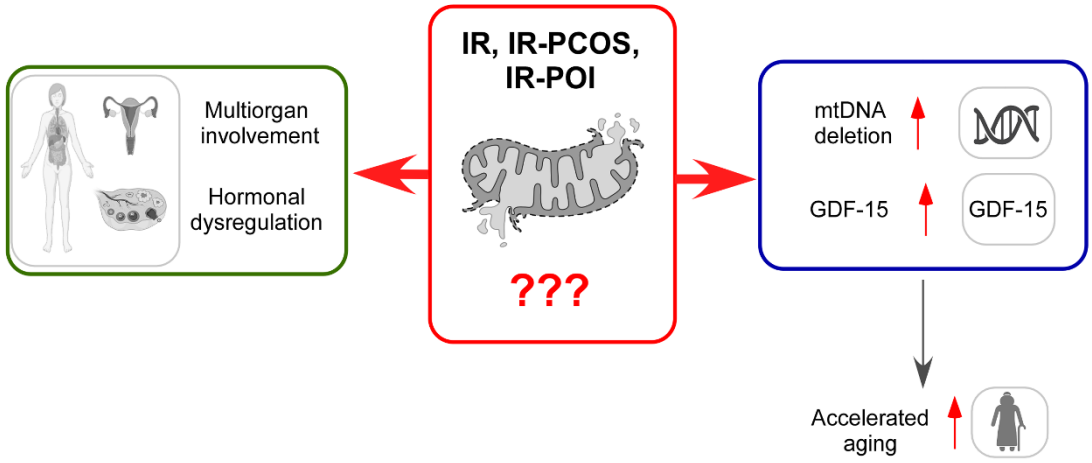

Figure 11. Integrated summary of mitochondrial and metabolic alterations identified in the study (110).

Graphical summary of the key findings described in the present work. Metabolic disturbances associated with increased adiposity and insulin resistance are linked to markers of mitochondrial stress and genomic instability. Elevated GDF-15 levels and accumulation of mtDNA deletions are proposed as indicators of mitochondrial dysfunction, potentially contributing to impaired carbohydrate metabolism and features of accelerated biological aging. The diagram illustrates hypothesized relationships rather than direct causal associations.

6. CONCLUSIONS

- Mitochondrial dysfunction may represent a unifying biological framework linking insulin resistance with PCOS and POI.
- Elevated plasma GDF-15 and mtDNA deletions identify a characteristic mitochondrial stress phenotype in insulin-resistant women.
- GDF-15 correlates with metabolic burden (BMI, reactive hyperinsulinemia, insulin dynamics) and reflects therapeutic demand.
- MtDNA deletions were associated with a steeper age-related decline in ovarian reserve markers in this cross-sectional cohort, reflected by lower AMH/FSH ratios and a more pronounced reduction in AMH with age, suggesting a potential link between mitochondrial genome instability and reproductive aging dynamics.
- Mitochondrial genome instability is linked to multisystem involvement, extending beyond reproductive dysfunction.
- These findings support the role of mitochondrial biomarkers in risk stratification, metabolic management, and fertility counseling (Figures 10–11).

7. SUMMARY

Aims/hypothesis: This thesis investigated the role of mitochondrial dysfunction in insulin resistance (IR) and its associated female reproductive endocrine disorders, with a particular focus on polycystic ovary syndrome (PCOS) and premature ovarian insufficiency (POI). It was hypothesised that GDF-15 and mtDNA deletions act as complementary biomarkers of mitonuclear stress, reflecting metabolic burden and contributing to accelerated reproductive and systemic aging.

Methods: Two observational studies were conducted, including 81 insulin-resistant women with or without PCOS or POI, as well as healthy controls. The participants were divided into three subgroups: IR-only, IR-PCOS, and IR-POI. Insulin resistance was defined using oral glucose tolerance testing in accordance with national guidelines. Plasma GDF-15 levels were measured using an enzyme-linked immunosorbent assay, and mtDNA deletions were detected via long-range polymerase chain reaction. Reproductive and thyroid hormones, metabolic indices, and multisystem clinical symptoms were also assessed.

Results: Plasma GDF-15 levels were significantly higher in insulin-resistant patients than in controls, and were associated with body mass index and metabolic severity. MtDNA deletions were most prevalent in the insulin resistance (IR)-only subgroup, suggesting that mitochondrial impairment may precede overt reproductive dysfunction. Elevated GDF-15 levels and mtDNA deletions were associated with multisystem clinical features and endocrine alterations. Free thyroxine emerged as an independent predictor of GDF-15. Reduced AMH/FSH ratios and age-related alterations in AMH, particularly in women with mtDNA deletions, indicated impaired ovarian reserve and accelerated reproductive aging.

Conclusion: These findings suggest that mitochondrial dysfunction is a key biological mechanism that links insulin resistance to systemic manifestations and reproductive endocrine decline. GDF-15 and mtDNA deletions act as complementary biomarkers of mitonuclear stress, reflecting metabolic load and the onset of aging processes. Together, they may help to identify women at increased risk of reproductive impairment and accelerated aging, which could have implications for personalised metabolic and reproductive management.

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