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Sample References

Papers Published in Periodical Journals: Gungor N, Saad R, Janosky J, Arslanian S. Validation of surrogate estimates of insulin sensitivity and insulin secretion in children and adolescents. J Pediatr 2004;144:47-55.

Papers Only Published with DOI Numbers: Knops NB, Sneeuw KC, Brand R, Hile ET, de Ouden AL, Wit JM, Verloove-Vanhorick SP. Catch-up growth up to ten years of age in children born very preterm or with very low birth weight. BMC Pediatrics 2005 doi: 10.1186/1471-2431-5-26.

Book Chapters: Darendeliler F. Growth Hormone Treatment in Rare Disorders: The KIGS Experience. In: Ranke MB, Price DA, Reiter EO (eds). Growth Hormone Therapy in Pediatrics: 20 Years of KIGS. Basel, Karger, 2007;213-239.

Books: Practical Endocrinology and Diabetes in Children. Raine JE, Donaldson MDC, Gregory JW, Savage MO. London, Blackwell Science, 2001;37-60.

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Review

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10-12 September 2016, Paris, France

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ECE 2017 (19th European Congress of Endocrinology)
20-23 May 2017, Lisbon, Portugal

ECO 2017 (24th European Congress on Obesity)
17-20 May 2017, Porto, Portugal

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Review

Environmental Contaminants and Pancreatic Beta-Cells

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**in memoriam*

ABSTRACT

Despite health policies as well as clinical and research efforts, diabetes prevalence is still rising around the world. A multitude of causes have been suggested for this increase, mostly related to familial background, the occidental diet which is rich in fat/carbohydrates, and sedentary life style. Type 2 diabetes involves malfunctions of the primary pancreatic beta-cells, usually attributed to local damage; however, it can be associated with other stressful environmental agents, such as chemical contaminants from food, plastic and air, among others. Indeed, exposure to these chemical agents during perinatal and adolescent life can increase the risk of developing cardiometabolic diseases later in life. This review explores data showing which environmental chemical agents may produce injury in beta-cells and further impair the insulinotropic process of type 2 diabetes. Additionally, it points the need to also consider unusual causes of metabolic diseases, such as environmental contaminants.
Keywords: Contaminants, pancreatic beta-cell, diabetes, insulin resistance

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Introduction

Type 2 diabetes is becoming a great problem for world health authorities, and it is generally accepted that a sedentary lifestyle, an increase in high-fat diets, and genetic factors do not completely explain this epidemic, whose origin may be in the maternal womb (1). Among all the functions of the pancreas, insulin production by beta-cells has received great attention due to their functions related to glycemia control, which is an important hallmark of metabolism control. Beta-cells are very sensitive and are the only cell type that can produce, store, and release insulin; when they fail or are destroyed or affected, the metabolism is impaired and the onset of diabetes may occur (2). The endocrine pancreas is formed by pancreatic islets, also

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termed Langerhans islets, consisting of four different types of endocrine cells known as the beta, delta, alpha, and pancreatic polypeptide (PP) cells which produce, store, and release insulin, glucagon, somatostatin, and pancreatic peptide, respectively. Islets also occur in lower quantities in the acinar structures of the pancreas. Although beta-cells occupy a great part of the volume in the islets, their capacity to proliferate and their neo-formation are extremely reduced compared to other cells (3). Insulin secretion control is distinct from all other cells that release hormones or neurotransmitters; glucose, as a major physiological stimulus, induces very high cellular metabolism activity, promoting the production of adenosine triphosphate (ATP) which can block a specific potassium (K+) channel. Once K+ output is reduced, the beta-cell is depolarized which induces certain calcium (Ca^{2+}) channels to open, permitting a rise in intracellular Ca^{2+} (1). The cytoskeleton is activated, stimulating the transport of vesicles that contain insulin to the periphery of plasma membrane, where exocytosis is performed and the insulin is released. Save for a few neurons in the hypothalamus, beta-cells can be considered a unique metabolic sensor. Other secretory cells are signalized using mostly a membrane receptor (4).

The high incidence of metabolic diseases such as type 2 diabetes cannot be explained solely by the occidental diet and sedentary life style. In the last 10 years, it has been shown that the influence of environmental pollutants such as food contaminants, plasticizers, pesticides (Ps), and organic metallic compounds might contribute to the early onset of obesity and beta-cell malfunction which allows the development of diabetes (5). Environmental contaminants injure pancreatic beta-cells impairing the metabolism; however, when the exposure is halted, the beta-cells might recover improving the metabolism. Nevertheless, exposure to contaminant during perinatal life can damage the beta-cells disrupting fetal metabolism, a state which can persist even in adulthood (1).

There is a theory that certain challenges during gestation, mostly malnourishment, including a restricted caloric diet, that stress the fetus in the maternal womb can compromise growth and development. In this condition, babies are delivered with low birth weight and are at high risk for cardiometabolic disorders such as obesity, hypertension, and diabetes (6). The stressed fetus adopts strategies to maximize its chances for survival and growth in postnatal life. Adapting its metabolism to live in restricted caloric conditions is one such strategy. These survival maneuvers promote the maintenance of noble tissues, such as the brain and heart, but can compromise that of others such as the liver, muscles, and pancreas. However, upon exposure to food abundance, the metabolism saves the excess and overaccumulates energy stocks. This concept, which emerged in the 1990s, is known as developmental origins of health and disease (DOHaD) but also termed the Barker theory, the thrifty phenotype theory, and metabolic programming, among others (7). Interestingly, in the 2000s, a theory that assigns environmental chemicals which affect adults as well as fetuses has emerged. According to this theory, environmental chemicals are the cause of the cardiometabolic disruption pandemic worldwide (8). During gestation, to maintain the development of the fetus, hormone and nutrient blood levels, including insulin, leptin, estrogen, and glucose, show an increase (1). Thus, the muscles and adipose tissues of the mother reduce their glucose uptake, building an insulin-resistant framework for a specific time (9). However, when insulin secretion/insulin sensitivity is impaired, an increase in insulin resistance is triggered, inducing gestational diabetes mellitus (GDM) which in turn is linked to high birth weight, early onset of obesity, and type 2 diabetes in adulthood (1).

Specifically the first and third parts of gestation are critical periods in the development of pancreatic islets in humans and rodents, respectively (2). The end of this process, whereby the function of the pancreatic islets are established, occurs during the lactation period (10). Regarding pollutants and precocious diabetes, there is strong evidence that environmental pollutants are present in the mother's placenta and can be transferred to the fetus producing diabetes in later stages of life (8). However, the mechanisms by which pollutants might program the fetus to develop diabetes in adulthood are not yet clear or fully understood. This brief review aims to help understand the relationship between precocious exposure to environmental pollutants and pancreatic beta-cell damage and the possible role of this relationship in type 2 diabetes onset.

Food Contaminants and Diabetes

Chemicals or pollutants that are generally called endocrine-disrupting chemicals (EDCs) act directly on the function of endocrine system and may inhibit the release and action of several hormones related with body metabolism (11). Originally, these chemicals were thought to act primarily through nuclear hormone receptors, including estrogen receptors (ERs), androgen receptors (ARs), progesterone receptors, thyroid receptors (TRs), and retinoid receptors, among others, but with increasing knowledge, the mechanisms of action have been recognized as much broader, and an EDC is now accepted as any compound, either natural or synthetic, that, through environmental or inappropriate exposure, alters the hormonal system (12). EDCs include industrial contaminants, plasticizers, food contaminants, polychlorinated biphenyls (PCBs), metals, Ps, and other chemicals that can be accumulated in body tissues, mainly in adipose tissue due to their lipophilic nature (13). It is accepted that the EDCs, since they have a great affinity for ERs alpha and beta ($ER\alpha$, $ER\beta$), can impair the hormonal system mainly during gestation, leading to deregulation of functions related to sexual differentiation as well as affecting insulin sensitivity and production in the mother and the fetus (5).

As commented above, these compounds can deregulate body metabolism. However, it must also be said that EDCs act in different ways which vary by range of dose, time of exposition, and the particular metabolic pathway. In other words, an individual, following exposure to one of these compounds, may or may not show a response (14). The relevant literature indicates that in humans, the EDCs may produce biological changes in doses lower than the allowed lowest doses, producing U shaped or inverted-U shaped dose–response curves. These so-called nonmonotonic doses represent a response with a change in the sign to positive for negative or vice and versa in the slope over the dose range tested. The PCBs can act as an agonist or antagonist and inhibit

the hypothalamic-pituitary-thyroid axis (HPA) or even lead to impairment of some TRs and affect thyroid hormone signaling and action (15). Although the EDCs are claimed to elicit obesity by acting directly on white adipose tissue, other structures such as brain, liver, the endocrine pancreas, and especially pancreatic beta-cells, may be direct targets as well (14). Taken together the above-presented evidences, the sections of this review aim to show that exposure to any type of EDC may possibly lead to changes in the structure or function of pancreatic beta-cells and play a role in type 2 diabetes onset.

Xenoestrogens and Estrogens

During gestation, regardless of development of GDM, insulin resistance increases due to the requirement of the fetus for glucose (16). The increase of estrogen appears to be linked to insulin secretion and sensitivity as well as to GDM (5). In fact, several studies show that during pregnancy, the pancreatic islets adapt to the high insulin demand. It has been shown that the islets are high in size and number and that proliferation and neogenesis are dramatically increased in beta-cells during this period (17). In the past, this increase was attributed to lactogenic hormones; however, estrogen is currently associated with the increases in insulin secretion and sensitivity. This sexual hormone promotes a protective effect against oxidative stress and pro-inflammatory cytokine-induced apoptosis in pancreatic beta-cells (1).

In support of this point, it is known that low levels of estrogen in ovariectomized or aged rats are associated with glucose intolerance, insulin resistance, dyslipidemia, and obesity but, interestingly, it was shown that an excess of estrogen also induced metabolic disruption causing obesity (18). Beta-cells present ERs (ERα and ERβ) (1). Indeed, knockout mice to estrogen alpha-receptor (ERαKO) exhibit a reduction in glucose transporter type 4 (GLUT4), while knockout mice to estrogen beta-receptor (ERβKO) present no differences although it is well documented that ERβ is involved in body fat distribution (5). These observations and comments provide evidence that estrogen can influence beta-cell function, and unexpected estrogen fluctuation levels might contribute to metabolic diseases.

Within this context, it has been accepted that estrogens and xenoestrogens such as bisphenol A (BPA), a contaminant associated with plastic packs, may stimulate high production of estrogen in both females and males with substantial effects (19). In fact, estrogen elevation induces a rise in Ca^{+2} in beta-cells, due to closure of the K_{atp} channel, provoking an increase in insulin secretion (16). This same study also shows that not ERα but ERβ mediates rapid estradiol effect, and thus, in synergy with glucose, when the estradiol binds to ERβ, the guanylate cyclase receptor is activated through an unknown pathway. As a consequence, K_{atp} channel activity decreases in a cGMP-dependent protein kinase (cGMP/PKG) in a dependent manner, and this effect finally potentiates enhanced insulin secretion (20). Moreover, in rats that received anti-estrogens, the content of insulin was decreased (1). However, it is important to emphasize that estrogen causes insulin resistance, which in turn provokes insulin oversecretion. Estrogen is also associated directly with insulin release from beta-cells. Thus, estrogen leads by different pathways to beta-cell exhaustion, death, and inhibited proliferation, allowing the onset of type 2 diabetes (16).

Persistent Organic Pollutants

Regarding the growth of the type 2 diabetes epidemic, many studies have indicated the influence of persistent organic pollutants (POPs). Exposure to this type of pollutant during gestation and lactation causes glucose intolerance in the offspring, supporting the existence of a potential mechanism for triggering adulthood diabetes (21). POPs are highly spread in the environment and in high doses can provoke toxicity in animals and human beings. Mainly, this class of contaminants impairs neuroendocrine functions (22).

Among POPs, it is known that the contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), used as a herbicide (no more commercialized applications), is one of the most toxic. Its effects are exerted through the activation of a specific cytosolic receptor, the aryl hydrocarbon receptor (AhR) (21). TCDD exposure in animals is related to wasting syndrome, characterized by loss of body weight or reduced weight gain due to difficulty in using blood nutrients, a condition also associated with hyperglycemia (23). TCDD also reduces glucose uptake in the adipose tissue, pancreas, and liver (22).

Cell death due to an increase in pro-inflammation cytokines is observed in insulin secreting cells (INS-1E) when exposed to low doses of TCDD (12,5 and 25 nM) for only 1 hour (24). Thus, beta-cells may be an important target for the action of this contaminant. In fact, TCDD has been observed to stimulate autophagy in cultured cells (25). In advanced stages of type 2 diabetes, autophagy in beta-cells occurs due to increased inflammation signals contributing to the decrease in insulin secretion (26).

Another pathway for increased diabetes onset mediated by TCDD is TCDD-induced (Ca^{+2}) influx via calcium T-type channels that regulate vesicular trafficking, such as lysosomal and secretory granule exocytosis. According to the increase in Ca^{+2} is linked to beta-cell depolarization and thus related to reduced K_{atp} channel activity and increased insulin secretion and the possible exhaustion of beta-cells (22).

Metals

Diabetes is widely explained as a physiopathological situation involving difficulty in promoting glucose uptake by peripheral tissues, mainly due to impaired insulin secretion and/or low insulin sensitivity in the glucose uptake tissues (27). It has been accepted that with advancing age, this diabetic framework may worsen, and pollutants with long half-lives such as some metals may be involved in this progression (28).

Cadmium is a non-essential toxic metal that is highly present in the environment (29). Other than tobacco, diet is the major source of Cd and it is found in cereals, potatoes, and root vegetables. It has been identified as a metallo-estrogen (30). It has been observed that Cd gradually accumulates in beta-cells. Studies show that Cd has a half-life of 30 years. Indeed, long-term Cd exposure induces a reduction in insulin secretion (28). However, the mechanisms involved in beta-cell failure due to Cd exposure are not known. One study suggested reduced calcium uptake by the pancreatic beta-

cells (31), but further studies are needed to determine the correct pathways behind Cd and type 2 diabetes.

In addition to Cd, arsenic (As) is also known as a potential stressor of pancreatic cells, and the main source of contact is contaminated water (32). It is known that As stimulates autophagy maneuvers due to increased reactive oxygen species (ROS). Zhu et al (33) showed that when insulin-secreting (INS-1) cells are exposed to low doses of As (1-4 μM) for 24 hours, the production of ROS was increased, which was related to cell death due to high autophagy and resulted in reduced insulin secretion. Alternatively, it has been shown that the transcription of insulin genes such as *Pdx1*, a gene involved in pancreatic cell maturation and survival, is downregulated by As exposure (34).

Last but not least, mercury (35) is also related to glucose intolerance (36). However, much of what is known about its toxic effects is related to nervous system development (37). The major source of Hg is seafood consumption (38). In relation to pancreatic beta-cell failure, it is reported that Hg in low doses can induce cell stress and cellular death and cause pancreatic islet beta-cell dysfunction which may lead to diabetes development (37,39). In rodents, a low dose of Hg was able to induce an increase in oxidative stress leading to signaling pathway activation of phosphatidylinositol 3-kinase (PI3K)-Akt, which was related to impaired insulin secretion (39).

However, not only these metals but, according to other studies, excess or deficiency of certain essential trace metals such as zinc and nickel may play an important role in beta-cell malfunction. In fact, available evidence shows that these elements are found in large quantities in diabetic persons, however, more studies are needed to show this relationship and the possible pathways involved (37).

Pesticides

Exposure to Ps has been related to neurotoxicity, but there is a growing body of evidence indicating that Ps can induce metabolic diseases, such as obesity and type 2 diabetes (40). In fact, Ps are considered carcinogenic EDCs interacting with estrogens mainly via the thyroid hormones and increasing the expression of estrogen-responsive genes (41). Ps, mainly organophosphates, attack neuron connections via blocking acetylcholinesterase (AChE) activity but can also induce pancreatitis, thus damaging beta-cells. Moreover, hyperglycemia is frequently associated with exposure to Ps (40). Although little is known about how Ps can induce hyperglycemia, it is suggested that the inhibition of AChE activity may increase ROS and lead to high cell death (41).

Malathion, a specific pesticide, is used as an insecticide in agricultural, veterinary, medical, and public health practice (41). It has been associated with metabolic disorders such as obesity and diabetes (42). In relation to type 2 diabetes, malathion causes an increase of intracellular Ca^{+2} in beta-cells, which could cause a loss of Ca^{+2}/calmodulin-dependent protein kinase function that is involved in the regulation of insulin secretion (41). Moreover, malathion is associated with increased apoptosis due to toxic effects on islet mitochondria thus increasing ROS production.

Another pesticide, diazinon, which is also employed in agricultural practice, is associated with impairment in glucose uptake and insulin secretion. Pakzad et al (42) suggested that the pathway of this metabolic disorder is mainly through the stimulation of muscarinic receptors which are involved in the process of glucose-induced insulin secretion. As an acetylcholine agonist, diazinon potentiates glucose-induced insulin secretion. High doses and/or long-term exposure to this pesticide might exhaust pancreatic beta-cells, allowing diabetes onset (42,43). In other words, exposure to Ps can result in increased AChE overstimulation, down regulation of muscarinic receptors, and most likely reduced production of insulin, similar to findings in rats exposed to a high-fat diet and mono sodium L-glutamate (43,44). In results not yet published, our laboratory has shown that perinatal exposure to acephate, another organophosphate pesticide, provokes beta-cell malfunction in the adult life of rat offspring. Collectively, all this information gives clear evidence associating pesticide exposure and type 2 diabetes, but further studies are needed for accurate determination of the pathways involved.

Food Processing Products

It is known that environmental pollutants are linked to the increasing diabetes epidemic and that reactive species accepted as contaminants are produced in food storage or preparation. Examples include advanced glycation end products (AGEs) of the Maillard reaction (45). More specifically, the Maillard reaction is a reduction reaction of carbohydrates with amino compounds which is responsible for the aroma, taste, and appearance of thermally processed food (46). The best-known AGE products are methylglyoxal (MG), acrylamide (AA), and N(ε) -(carboxymethyl) lysine (CML) which in elevated levels are associated with glucose intolerance and insulin resistance (45).

Excess glucose has been accepted as a precursor of beta-cell damage receiving the name of glucotoxicity (47). In fact, this toxicity due to hyperglycemia, in the long term, can increase the levels of ROS and endoplasmic reticulum (ER) stress, which are related to high levels of Ca^{+2} and the occurrence of the glycation reaction (48). The glycation reaction has been linked to the irreversible and heterogeneous production of species or products from reactive dicarbonyls, in other words, the non-enzymatic linkage of glucose with an amino group; the products of this reaction are termed AGEs (47).

The main source of AGEs is an increase in intake of thermally processed foods. Sustained hyperglycemia is one other cause (glucotoxicity) (48). Glucotoxicity is identified as an important characteristic of problems related to beta-cell failure and type 2 diabetes because it contributes to increased ROS production, ER stress, and intracellular and extracellular AGEs formation (49). The action of AGEs is triggered by the linkage between an AGE and the receptor termed advanced glycation end products receptor (RAGE) (50). The AGE products overstimulate RAGE resulting in decreased glucose-stimulated insulin secretion and high cell apoptosis due to the elevation of ROS (48). Pancreatic islets isolated from normal adult rats with MG for 24 hours show an altered insulin secretion response to glucose, which suggests beta-cells as a target of AGE substances (51).

What is the Importance of Endocrine Disrupting Chemical Mixtures?

In the previous sections, information was given about the role of different contaminants in the progression of the diabetes type 2 epidemic, and the need to focus efforts on developing therapies for reducing this framework was brought to attention. However, it is important to emphasize that the individual is in constant lifelong contact with the environment, continuously receiving different stimuli. Thus, it is likely that many EDCs are acting together because environmental contamination is rarely due to a single compound, and the effects of different classes of EDCs may be additive or even synergistic (12).

Normally, humans or animals are exposed to a great variety of known and unknown contaminants throughout life, and individuals have differences in metabolism, body composition, and gene expression that can increase or decrease the half-life of pollutants, which may or may not produce their potential effects (52). Regarding metabolic disorders, it is likely that the progression of these diseases is the result of chronic exposure to mixtures of low amounts of EDCs, and the latency between pollutant exposure and clinical disorders creates further challenges in attempting to establish a relationship to the level of exposure and the physiology of each person (12).

Metabolic Programming and Food Contaminants

Regarding the formation of pancreatic islets in rodents, it is known that at 14 days of gestation, in response to the influence of different growth factors, hitherto non-differentiated stem cells promote the emergence of different cell types representing a precocious maturing stage of pancreatic islets. In humans, the same process occurs in the first trimester of gestation, more specifically in the first ten weeks of life (53,54). In rodents, the immature pancreas presents a few cells that produce glucagon, which appears to be responsible for stimulating the initial production of insulin-secreting cells; however, this stage does not yet represent the true insulin secretion. In humans, insulin secretion begins during gestation, but in rats, it occurs only during lactation (53,55). Within the pancreatic islets formation process, several transcription factors are indispensable for promoting maturation and defining the future function of the cells. The expression of many transcription factors, such as paired box Pax4 and 6, homeodomain (Nkx) 6.1 and 2.2, fork head box (Fox) O1 and A2, neurogenin (Ngn) 3, and pancreatic and duodenal homeobox-1 (Pdx-1), among others, contribute as crucial markers in the pancreatic beta-cells for growth and survival throughout life (27).

Among all the functions of insulin, the most essential is ensuring that sugar is correctly taken up and stored in peripheral tissues such as the liver, muscles, and adipose tissue (13). Adequate glucose sensitivity, beyond all other nutrients, rapidly regulates insulin synthesis and secretion and is also critical for the maintenance of the glucose-responsive state and the number of beta-cells (40). During the development of the pancreas as well as subsequently, beta-cell apoptosis is normal, although this loss is compensated by neogenesis from preexisting beta-cells or from trans-differentiation of acinar cells (56). Thus, it is important to emphasize that outside the period during which the pancreas is formed, the amount of beta-cells may increase or decrease, and this process is dependent on how beta-cell formation occurred, in other words, if it was stressed or not (57).

Regarding precocious exposure to EDCs, since the studies of Barker about thrifty phenotype, more recent studies regarding another theory following the same principle have shown that early nutritional insults combined with exposure to environmental pollutants promoted an increase in obesity and related diseases and may have an influence on the formation of the pancreatic islets. In fact, it is accepted that during fetal life, the individual is more susceptible to environmental insults and these insults may lead to irreversible gene expression alterations (13,58).

Metabolic programming develops epigenetic modulation, which is defined as heritable changes in gene expression that are not due to any alteration in the primary DNA sequence. Epigenetic mechanisms include DNA methylation, histone modification, and regulation by noncoding RNAs. The way the genome interacts with and responds to the environment and even potentially the way the genome can influence its own environment via its effects on behavior are controlled by epigenetic changes (59). Epigenetic changes, particularly in DNA methylation, provide a "memory" of developmental plastic responses to the early environment and are central to the generation of phenotypes and their stability throughout life (13). As shown in this review, it is known that when estrogen action occurs at an inappropriate time or at non-physiological levels, adverse effects such as insulin resistance and hyperglycemia may occur. In addition, when glucose tolerance is impaired during pregnancy, the levels of DNA methylation in the genes involved in islet development may be modified, allowing reduced expression; in fact, the metabolism of early embryos is affected by maternal hyperglycemia, which causes the down-regulation of embryonic genes related to insulin action, such as GLUT 1,2, and 3 in the blastocyst stage (60).

In accordance with these points, Lin et al (61) showed that maternal exposure to Bis(2-ethylhexyl) phthalate (DEHP) promotes the reduction of important genes involved in beta-cell development. The authors showed that *Pdx-1*, a gene involved in the origin of pancreatic islets formation, was downregulated in the offspring of mothers exposed to DEHP during gestation. *Pdx-1* plays an important role in islet formation and gene transcription, and thus the reduction or even absence of this gene impairs insulin production and insulin output. In other words, the reduction in beta-cells provoked by DEHP during perinatal life, through decreased *Pdx-1* gene expression, compromises the beta-cell function of the offspring (61). Moreover, downregulated *Pdx-1* is also related to impaired mitochondrial function (62). During pancreas formation, beta-cells have high energy demand and poor antioxidant defense, so when mitochondrial function is decreased, an increase in ROS occurs promoting damage to mitochondrial DNA that is associated with the emergence of diabetes in adulthood (55).

To conclude, it is widely accepted that the fetal period is critical for the development of healthy beta-cells in adulthood. The food intake behavior and environment of the mother may program the child for future health and unfortunately, future diseases. Not all pathways for this malprogramming are known, but new knowledge regarding the relation between contaminants and

diseases can greatly aid in eliminating the metabolic pandemic, saving future lives.

Ethics

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Gabriel Fabricio, Ananda Malta, Design: Gabriel Fabricio, Ananda Malta, Data Collection or Processing: Gabriel Fabricio, Ananda Malta, Analysis or Interpretation: Abalo Chango, Paulo Cezar de Freitas Mathias, Literature Research: Gabriel Fabricio, Ananda Malta, Abalo Chango, Paulo Cezar de Freitas Mathias, Writing: Gabriel Fabricio, Ananda Malta.

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Original Article

The Relationship between Maternal Gestational Impaired Glucose Tolerance and Risk of Large-for-Gestational-Age Infant: A Meta-Analysis of 14 Studies

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ABSTRACT

Objective: To explore, by conducting a meta-analysis, whether gestational impaired glucose tolerance (IGT) is an independent predictor of neonatal large for gestational age (LGA) or not.

Methods: Medline, Embase, and Cochrane Library databases were searched to identify published epidemiological studies (cohort and case-control studies) investigating the association between gestational IGT and neonatal LGA. Calculations of pooled estimates were conducted in random-effect models or fixed-effects models. Heterogeneity was tested by using chi-square test and I^2 statistics. Egger's test (linear regression method) and Begg's test (rank correlation method) were used to assess potential publication bias.

Results: Fourteen observational studies were included in the meta-analysis. The overall risk for the effect of IGT on LGA was 2.09 (1.56, 2.78). Stratified analyses showed no differences regarding different geographic regions or the analysis of overall adjusted odds ratios. No evidence of publication bias was observed in either Egger's test or Begg's test results.

Conclusion: Gestational IGT is an independent predictor of neonatal LGA.

Keywords: Gestational impaired glucose tolerance, large for gestational age, meta-analysis

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Some studies demonstrated that women with gestational impaired glucose tolerance were at higher risk of adverse pregnancy outcomes while compared with women with normal glucose tolerance, but some studies supported the idea that the discrepancies of the searches may be related to the criteria used to diagnose this condition.

WHAT THIS STUDY ADDS?

Gestational impaired glucose tolerance is an independent predictor of neonatal large for gestational age.

Introduction

Gestational impaired glucose tolerance (IGT) is defined as an abnormal glucose level obtained in an oral glucose tolerance test (OGTT) during pregnancy. Gestational IGT is considered to reflect a serious defect in beta-cell function in the early and

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late-phases of insulin secretion and is regarded as a sign of pre-gestational diabetes mellitus (GDM) (1). Universally, GDM is associated with adverse pregnancy outcomes and its incidence increased in parallel to the increase in frequency of obesity worldwide. Women identified as GDM patients are treated with dietary or insulin therapy to reduce their glucose levels and hence the risk of adverse pregnancy outcomes (2). While the importance of identification and treatment of GDM and the benefit of controlled blood glucose in the prenatal period is universally confirmed, knowledge on the mechanisms responsible for the impact of gestational IGT on pregnancy outcome is inconclusive. The offspring of women with IGT, compared to those of women who had good glucose control during pregnancy, are reported to have increased birth weights, increased rates of macrosomia, and increased frequency of large for gestational age (LGA) (3). Some studies demonstrated that women with gestational IGT were at higher risk of adverse pregnancy outcomes as compared with women with normal glucose tolerance (NGT), but others have attributed these findings to differences in the criteria used to diagnose this condition (4,5). However, there is no systematic review or meta-analysis of the studies on the importance of gestational IGT as a public health problem. With this background, we attempted to conduct a meta-analysis of the studies on the association between gestational IGT and pregnancy outcome published within the last decade.

Methods

We performed a detailed search on Medline, Embase, and Cochrane Library to identify articles that reported the relationships between IGT during pregnancy and neonatal outcomes. We also attempted to reach the comments on these studies through review articles. The database was searched from 1999 to April 2015 and limited to human studies which were published in English.

We used the following search terms: "gestational" or "pregnant" and "impaired glucose tolerance" or "IGT" and "large for gestational age" or "LGA". Studies were included in the analysis if they examined outcomes in pregnant women who had IGT but not GDM and women who had not received any treatment. The primary adverse outcome searched in this meta-analysis was LGA, defined as a birth weight >90th percentile for gestational age.

Quality assessment of the available studies was conducted independently by two reviewers (Hai-Qing Wang, Han-Lin Lai) using the Newcastle-Ottawa quality assessment scale for cohort studies and for case-control studies (6). The scores range from 0 to 9 and scores ≥6 were graded as of high-quality.

Data Extraction

Using a standardized data-collection form, the two reviewers (Hai-Qing Wang, Han-Lin Lai) extracted the data from the searched article independently, and any disagreement was resolved by discussion. The following study characteristics were recorded: first author's name, year of publication, country of origin, study design, inclusion and exclusion criteria, sample size, diagnostic criteria for gestational IGT, potential confounding factors adjusted for. All search results were exported to Endnote 7.0 to organized references and duplications were thus eliminated.

Statistical Analysis

We extracted the odds ratio (OR) and the 95% confidence intervals (95% CI) to reflect the uncertainty of point estimates from each study. The crude OR for gestational IGT and LGA could be calculated from 5 studies and the other 9 studies which were stratified by some confounding factors (such as quality grade, number of confounding factors adjusted for, study population) which reported adjusted OR and the 95% CI. The chi-square test was used to analyze the heterogeneity of the results, and $p<0.10$ was considered as the cut-off level of heterogeneity. We also used I^2 to judge the heterogeneity between these studies, I^2 representing the percentage of the true heterogeneous (non-sampling error) in the total variability; when I^2 was >50%, we recognized the existence of heterogeneity (7). When substantial heterogeneity was detected, the summary estimate on the basis of the random-effects model using the method of Der Simonian and Laird (8) was presented. These two approaches yield similar results when the heterogeneity of the study is small, the random-effects model gives more weight to imprecise (or small) studies compared to a fixed-effects model (9). In addition, the pooled estimate that was based on the fixed-effects model using the inverse variance method was presented (10). In order to assess the impact on the results of a single study, we conducted a sensitivity analysis of each study by excluding each study one by one and recalculating the combined estimates on remaining studies. We used a funnel plot (11) to visualize the publication bias and used Egger's test (linear regression method) (12) and Begg's test (rank correlation method) (13) to assess potential publication bias. The Egger's test is a linear regression method about standard normal deviate and precision of all the studies in meta-analysis. The Begg's test is a rank correlation test for inspection of the correlation of effect and sample size. When the number of the studies in the meta-analysis is <20, the effects of these two methods are low, but the sensitivity of the Egger's test is higher than the Begg's test. Meta-analysis was performed with Stata/SE10.0 (Stata Corp, College Station, TX, USA).

Results

In the preliminary literature search, we identified 1377 unique citations from the electronic databases (Figure 1). No supernumerary article was found in the citations by

manual search and 145 were rejected because of duplicates. 711 were rejected because of 687 articles were on bias of titles, 6 studies were meta-analysis, and 18 were systematic reviews. The remaining 521 full-text articles were selected and inspected and then we excluded 507 articles because there were 30 reviews and 477 studies which did not meet the inclusion criteria of meta-analysis. Finally, we ended up with 14 observational studies (4,5,14,15,16,17,18,19,20,21,22,23,24, 25) for our analysis.

The characteristics of these 14 observational studies are displayed in Table 1. There were 13 cohort studies and only one case–control study. Six of the studies were conducted in Europe, 4 in North America, and 4 in Asia. The effect of gestational IGT on LGA and the definition of gestational IGT in each study are also demonstrated in Table 1.

The ORs of LGA in relation to gestational IGT from each study and the overall OR are presented in Figure 2. We assembled the OR and 95% CI of the 14 studies which

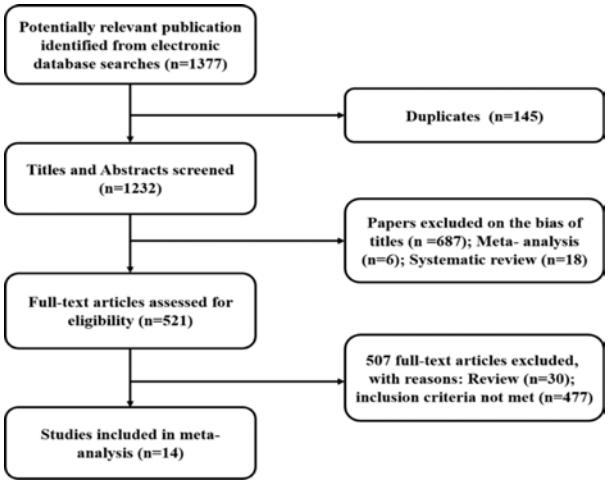

Figure 1. Process of literature search in our meta-analysis

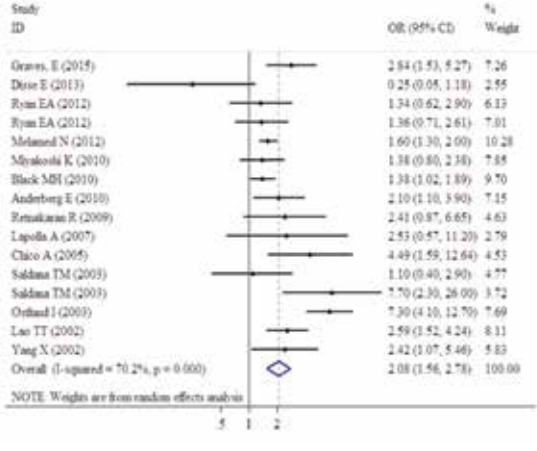

Figure 2. The odds ratio of large for gestational age infants in relation to gestational impaired glucose tolerance from individual studies and the overall odds ratio of these 14 observational studies

were related to the effect of gestational IGT on LGA, the homogeneity hypothesis was rejected by the chi-square test ($p<0.10$, $I^2=70.2\%$), thus we selected the random-effects model and obtained the overall OR, and 95% CI was 2.08 (1.56, 2.78) (Figure 2).

Table 2 presents the results of subgroup analyses of the effects of gestational IGT on LGA. When stratified by geographic region, a positive association of gestational IGT and LGA was observed in the studies conducted in each region. We abstracted the ORs from the 14 studies, the analysis of the effects of gestational IGT on LGA yielded an overall adjusted OR of 2.36 (1.64, 3.37), but this apparent relationship was not observed in the analyses of the unadjusted ORs. The definitions of gestational IGT in these studies were different - some studies restricted the value of fasting plasma glucose (FPG) (4,5,14,15,23,24), the others just formulated the value of OGTT (16,17,18,19,20,21,22,25). When stratified by the unequal definition, the analysis of the effects of gestational IGT with restricted FPG value on LGA yielded an overall OR of 1.73 (1.01, 2.99). The definition of gestational IGT employed different forms of OGTT as well - for instance, some studies used the value of OGTT at 0, 60, 120, and 180 min (19,20,21,25), some used the value of 2-h 75-g OGTT (4,5,14,15,17,18,22,23,24), and one used the value of 1-h 50-g OGTT (16). When we stratified by the different forms of OGTT, a positive association of gestational IGT and LGA was obtained in the studies conducted in unequal definition.

Sensitivity analyses investigating the influence of the 14 studies individually on the overall risk estimate by excluding one study per iteration suggested that the overall risk estimates did not substantially change by any single study. The analysis of the effects of gestational IGT on LGA was with a range from a low of OR 1.7 (95% CI 1.49, 1.95) to a high of OR 2.19 (95% CI 1.66, 2.9). The results did not change substantially after sensitivity analysis.

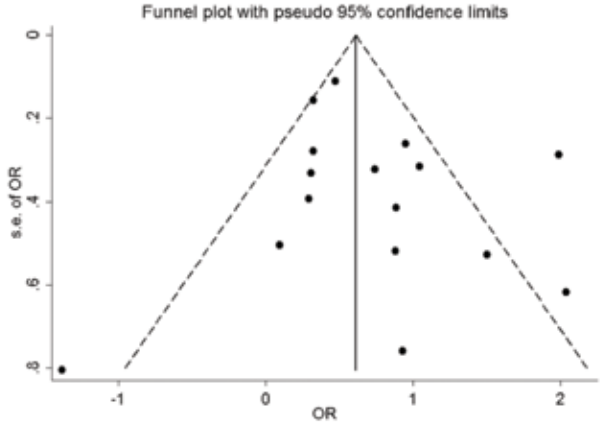

Figure 3. A funnel plot to visualize the publication bias of the 14 studies of this meta-analysis

Table 1. Characteristics of the studies included in the meta-analysis

Publication year, Author	Number of cases/ controls	Country	Study design	Effect on LGA	NOS scores[a]	Definition of IGT
2015, Graves et al (25)	26/2206	Canada	Cohort study	Adjusted OR 2.84 (1.53, 5.27)	6/9	50 g OGTT 0 time >5.2, 1 h >9.9, 2 h >8.5, 3 h >7.7 mmol/L, only one above the range
2013, Disse et al (14)	39/20	France	Cohort study	Unadjusted OR 0.25 (0.05,1.18)	8/9	FPG <5.1 mmol/L, 2-h 75 g OGTT >8.5 mmol/L
2012, Ryan (15)	104/368	Canada	Cohort study	All women: adjusted OR 1.36 (0.71,2.61) White women: adjusted OR 1.34 (0.62,2.90)	6/9	FPG <6.1 mmol/L, 2-h 75 g OGTT 7.8-11.0 mmol/L
2012, Melamed et al (16)	809/12,899	Israel	Cohort study	Adjusted OR 1.6 (1.3,2.0)	7/9	1-h 50 g OGTT > 7.7 mmol/L
2010, Miyakoshi et al (4)	174/4,512	Japan	Cohort study	Unadjusted OR 1.38 (0.8,2.38)	7/9	FPG <5.6 mmol/L, 2-h 75 g OGTT >8.3 mmol/L
2010, Black et al (5)	474/7020	USA	Cohort study	Unadjusted OR 1.38 (1.02,1.89)	7/9	FPG <5.1 mmol/L, 2-h 75 g OGTT >8.4 mmol/L
2010, Anderberg et al (17)	744/329	Sweden	Cohort study	Adjusted OR 2.1 (1.1,3.9)	8/9	2-h 75 g OGTT 8.6-9.9 mmol/L
2009, Retnakaran et al (18)	166/93	England	Cohort study	Unadjusted OR 2.41 (0.87,6.65)	7/9	2-h 75 g OGTT 7.8-11.0 mmol/L
2007, Lapolla et al (19)	48/334	Italy	Cohort study	Adjusted OR 2.53 (0.57,11.2)	7/9	100 g OGTT 0 m >5.2, 1 h >9.9, 2 h >8.5, 3 h >7.7 mmol/L, only one above the range
2005, Chico et al (20)	59/5767	Spain	Cohort study	Unadjusted OR 4.49 (1.59,12.64)	8/9	50 g OGTT 0 m >5.2, 1 h >9.9, 2 h >8.5, 3 h >7.7 mmol/L, only one above the range.
2003, Saldana et al (21)	White women 40/1,080 Black women 13/820	USA	Cohort study	White women adjusted OR 1.1 (0.4,2.9) Black women adjusted OR 7.7 (2.3,26)	7/9	100 g OGTT 0 m >5.2, 1 h >9.9, 2 h >8.5, 3 h >7.4 mmol/L, only one above the range
2003, Ostlund et al (23)	211/810	Sweden	Cohort study	Adjusted OR 7.3 (4.1,12.7)	7/9	FPG <6.7 mmol/L, 2-h 75 g OGTT 9.0-11.0 mmol/L
2002, Lao and Wong (24)	79/382	China	Case-control study	Adjusted OR 2.59 (1.52,4.24)	6/9	FPG <5.8 mmol/L 2-h 75 g OGTT >8.0 mmol/L
2002, Yang et al (22)	102/302	China	Cohort study	Adjusted OR 2.42 (1.07,5.46)	7/9	2-h 75 g OGTT 7.8-11.1 mmol/L

[a]: Study quality assessment is listed using the results of the Newcastle-Ottawa questionnaire. OR: odds ratio, OGTT: oral glucose tolerance test, FPG: fasting plasma glucose LGA: large for gestational age, NOS: Newcastle-Ottawa Scale, IGT: impaired glucose tolerance

Publication Bias

In the funnel plot (Figure 3), we found that the scatters are substantially symmetric. There was no evidence of potential publication bias with the association of gestational IGT with LGA, as suggested by Egger's test (p=0.314) and Begg's test (p=0.499).

Discussion

The aim of our meta-analysis was to explore the association between gestational IGT and LGA. The results of a total of 14 epidemiologic studies of this meta-analysis showed that gestational IGT is an independent risk factor for neonatal LGA. Egger's test and Begg's test revealed no significant publication bias. The overall adjusted OR indicated that gestational IGT is an independent risk factor for neonatal LGA, and the overall combined OR of the effects of IGT with restricted FPG value on LGA also reflected this conclusion. When we stratified IGT by the different forms of OGTT, the consequences of analysis implied that the different forms of OGTT employed in the studies have no effect on our conclusion. When we excluded one study per iteration, the range of variation of the overall is also smaller suggesting that no one study can significantly alter the findings.

Table 2. Sensitivity analysis of the effects of impaired glucose tolerance on large for gestational age

Group	Number of studies	OR (95% CI)	$p_{heterogeneity}$	I^2 (%)
Total	14	2.09 (1.56, 2.78)	<0.001	70.2
By geographic area				
North America	4	1.78 (1.17, 2.70)	0.04	57.0
Europe	6	2.52 (1.16, 5.47)	<0.001	75.7
Asia	4	1.71 (1.42, 2.05)	0.243	28.1
Adjusted OR	9	2.36 (1.64,3.37)	<0.001	71.7
Unadjusted OR	5	1.56(0.92,2.65)	0.034	61.5
Definition				
a	6	1.73 (1.01, 2.99)	<0.001	83.6
b	8	1.80 (1.50, 2.17)	0.105	41.0
c	4	2.89 (1.90, 4.38)	0.142	41.9
d	9	1.90 (1.26, 2.85)	<0.001	75.9
e	1	1.60 (1.30, 2.00)	-	-

[a]: Definition of impaired glucose tolerance based on restricted fasting plasma glucose value; b: definition of impaired glucose tolerance based on non-restricted fasting plasma glucose value; c: definition of impaired glucose tolerance based on the value of oral glucose tolerance test at 0, 60, 120, and 180 min; d: definition of impaired glucose tolerance based on the value of 75-g 2-h oral glucose tolerance test; e: definition of impaired glucose tolerance based on the value of 50-g 1-h oral glucose tolerance test. OR: odds ratio, CI: confidence interval

Gestational IGT is associated with postpartum metabolic dysfunction. Fetal growth in utero is a complex process and involves interactions among mother, placenta, and fetus. Mother's and fetal endocrine statuses, genetic predisposition, and available substrates result in fetal growth, all of which also determine birth weight. However, since the placenta does not allow transfer of insulin to the fetus, a large fraction of maternal glucose is metabolized in the fetus, leading to fetal lipogenesis and excessive growth (26). Therefore, it is conceivable that gestational IGT may contribute to fetal growth and future high birth weight.

In earlier studies, we found that the achievement of glucose control in women with at least one abnormal OGTT value decreased adverse neonatal outcomes to near baseline levels (27,28,29). In current studies, when we compared women with gestational IGT to those with NGT, we found that gestational IGT was associated with adverse perinatal outcomes (such as preterm birth) as well as with LGA and macrosomia (16,18,22,30). We also found that in women without gestational diabetes, gestational IGT is an independent predictor of having a LGA infant (15).

It has previously been reported that LGA is linearly related to maternal plasma glucose levels (31,32). Physicians have always been concerned about GDM but were unaware of gestational IGT as a pre-GDM condition, and women with gestational IGT were being cared for in the same way as normal pregnant women. As the results of our meta-analysis have shown, gestational IGT is an independent risk factor for neonatal LGA. Today, it is known that the monitoring of blood glucose during pregnancy is important for the control of the frequency of neonatal LGA. At this point, it is worth mentioning that recently, clinical studies have demonstrated that early intervention can prevent the development of diabetes in women with IGT (33). However, intervention trials of gestational IGT have not yet been realized in clinical trials. Therefore, if treatment suggestions are to be introduced to women with gestational IGT, the effects of such suggestions on pregnancy outcomes will need to be evaluated, also taking social, cultural, economic, and clinical benefits into account.

In conclusion, the results of our meta-analysis have shown that maternal gestational IGT increased the risk of LGA infants and was an independent predictor for neonatal LGA. Additional studies are needed to evaluate whether the monitoring of blood glucose and control of blood sugar by means of lifestyle programs (e.g. physical activity, diet) are beneficial in reducing the risk of neonatal LGA. The use of potentially biased evidence was the principal limitation of this study since the definition of gestational IGT showed differences among the studies. However, the consequences of the subgroup analyses implied that the different definition of gestational IGT employed in the studies had no effect on our conclusion.

Ethics

Ethics Committee Approval: Retrospective study, Informed Consent: Retrospective study.

Peer-review: External and Internal peer-reviewed.

Authorship Contributions

Concept: Li Li, Design: Li Li, Data Collection or Processing: Yi Li, Qi-Fei Liu, Shuang Hu, Analysis or Interpretation: Yi Li, Qi-Fei Liu, Shuang Hu, Literature Research: Hai-Qing Wang, Han-Lin Lai, Writing: Hai-Qing Wang, Han-Lin Lai.

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Original Article

Indices of Glucose Homeostasis in Cord Blood in Term and Preterm Newborns

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ABSTRACT

Objective: According to the thrifty phenotype hypothesis, intrauterine malnutrition has a role in the etiology of type 2 diabetes. This study was planned to determine the early alterations in indices of glucose homeostasis (glucose, insulin, and cortisol) in term and preterm newborns and the correlations of glucose, insulin, and cortisol levels with insulin resistance indices.

Methods: A descriptive study comprising 35 term and 35 preterm newborns was carried out from December 2013 to June 2015. Venous cord blood was collected and plasma glucose was analyzed by the glucose oxidase-peroxidase method in an auto analyzer. Serum insulin and cortisol levels were assessed by the enzyme-linked immunosorbent assay. Homeostasis model assessment of insulin resistance (HOMA-IR), quantitative insulin sensitivity check index and glucose insulin ratio were calculated to assess insulin resistance. The data on physical and metabolic parameters were analyzed using standard tests for statistical significance.

Results: In term newborns, mean glucose and cortisol levels (83.6±17.4 mg/dL and 11.88±5.78 µg/dL, respectively) were significantly higher than those in preterm infants (70.4±15.8 mg/dL and 8.9±4.6 µg/dL, respectively). Insulin and HOMA-IR levels were found higher in preterm newborns (10.8±4.8 µIU/mL and 1.52±0.66, respectively) than in term newborns (7.9±2.7 µIU/mL and 1.19±0.29, respectively). Insulin was found to positively correlate with HOMA-IR, whereas cortisol was negatively correlated with HOMA-IR in both term and preterm newborns.

Conclusion: Higher insulin levels and HOMA-IR values in the cord blood of preterm newborns support the theory of intrauterine origin of metabolic diseases.

Keywords: Cord blood, cortisol, glucose, insulin, insulin resistance

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Thrifty phenotype hypothesis states that the etiology of type 2 diabetes mellitus occurs early during intrauterine development. In few studies, term newborns' cord blood glucose, insulin, homeostasis model assessment of insulin resistance (HOMA-IR), and quantitative insulin sensitivity check index (QUICKI) were reported.

WHAT THIS STUDY ADDS?

As per our knowledge, for the first time in India, cord blood glucose, insulin, and cortisol levels in preterm and term newborns were estimated and insulin resistance was calculated using HOMA-IR, QUICKI, and glucose insulin ratio.

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Introduction

The newborn is considered to be in a transitional phase from a mother-dependent status to an independent-of-mother status. At delivery, the continuous transplacental flow of nutrients from the mother to the fetus stops abruptly. The successful transition from intrauterine to extrauterine life requires adaptation to several changes (1). Factors such as placental flow, maternal and hormonal secretion may determine and influence fetal growth (2). In this context, the hormones insulin and cortisol play an important role (3,4). Adaptation processes to extrauterine life are difficult to accomplish by the premature neonate. Thus, infants born prematurely are at higher risk for disturbed glucose homeostasis. They frequently develop hypoglycemia as a result of small substrate stores and immature enzyme systems (5). However, surprisingly, prevalence of hypoglycemia is estimated to vary between 40 and 80% among very preterm infants (6). This condition is much rarer in late preterm and term infants, highlighting the central role of immaturity in the pathophysiology of glucose homeostasis in preterm newborns (7).

Thrifty phenotype hypothesis regarding etiology of type 2 diabetes signifies that poor nutrition in fetal and early infant life is detrimental to the development and function of the β cells of the islets of Langerhans (8). The fetal insulin hypothesis also proposes a relationship between inherited insulin resistance and altered growth mediated by insulin (9). Early alterations in insulin and cortisol hormones influencing glucose homeostasis increase the risk of developing insulin resistance and obesity later in life (8). Thus, elevated insulin levels during perinatal life may predispose the infant to development of diabetes mellitus in future life (10).

The hormone concentration in the fetal circulation changes both developmentally and in response to nutritional stimuli. Near term, there is an increase and decrease in the concentrations of insulin and cortisol, respectively, signaling maturation of the fetus (11,12). An adrenocortical hormone, cortisol, is well-known as the stress-responsive hormone and its blood concentration is used as a stress marker. It modulates a large number of physiological actions involved in metabolic, inflammatory, cardiovascular, and behavioral processes. The molecular mechanisms and the physiological effects of cortisol have been extensively studied. However, the involvement of cord blood cortisol action in the etiology of diabetes and insulin resistance has not yet been clarified in term and preterm newborns. Recent mounting clinical evidence and animal studies have attracted growing interest in the role of cortisol action in obesity and insulin resistance (13).

Limited studies have been reported on glucose homeostasis indices, insulin resistance, and cortisol levels in cord blood of term and preterm newborns. Hence, this study was planned with an objective to determine the early alteration in indices of glucose homeostasis in cord blood of term and preterm newborns and the correlations of glucose, insulin, and cortisol with insulin resistance indices.

Methods

This cross-sectional study comprised 35 term and 35 preterm newborns who were born at the constituent medical college hospitals during the 1.5 years between December 2013 and June 2015. All infants were products of vaginal deliveries. The study was approved by the Institutional Ethics Committee and informed consent was obtained from the mothers. The study population comprised of offspring of residents of southern India mainly from in and around the city of Mangalore.

All selected term newborns were between 37 and 41 6/7 weeks of gestational age and their birthweight was between 2.5 and 4 kg. The preterm newborns were between 24 and 37 weeks of gestational age and their weights varied between 1.5 and 2.5 kg. Only term and preterm newborns with a 5^{th}-minute Apgar score >9 were included in the study. Mothers suffering from any infectious disease or having obstetric complications such as gestational diabetes, hypertension, kidney disease, thyroid disease, PCOD were excluded.

Venous cord blood (VCB) was collected under aseptic conditions from the umbilical cords of all 70 newborns. After delivery, but prior to expulsion of placenta, 3 mL blood was drawn from the umbilical cord into a plain and a fluoride vacutainer. Plasma glucose was determined within 4 hours of collection. The serum was stored at -20 ^{0}C until further analysis for insulin and cortisol by ELISA. All data regarding mother and newborn were collected from the hospital files.

Plasma glucose estimation was done by the glucose oxidase-peroxidase method (Agappe diagnostic kits, Ernakulam, Kerala) using a Roche Hitachi P800 auto-analyser (Roche Diagnostics GmbH, Mannheim). The coefficient of variation (CV) for intra- and inter-batch for glucose was <4%. Insulin levels were assayed based on sandwich principle in ELx 800 by BIO TEK® Instruments, Inc. using an insulin ELISA kit manufactured by DRG, a German company. The CV for intra- and inter-batch insulin assay was <3%. Cortisol levels were assayed based on sandwich principle in ELx 800 by BIO TEK® Instruments, Inc., using a cortisol ELISA kit manufactured by Cal biotech, a USA company. The CV for intra- and inter-batch insulin assay was <6%. Insulin resistance indices were calculated by three metabolic parameters. Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated using the equation: HOMA-IR=fasting insulin (μU/mL) x fasting glucose (mg/dL)/405 (14). Quantitative insulin sensitivity check index (QUICKI) and glucose insulin ratio (GIR) were calculated manually by calculation (15,16).

Statistical Analysis

The data were analyzed using the IBM SPSS Statistics version 20 (SPSS, Chicago, IL, USA). The parametric data were presented as means ± standard deviation (SD) and nonparametric data as medians (first quartile, third quartile). The student's (independent-samples) t-test and the Mann-Whitney U test were used to compare mean differences between the two groups for parametric and nonparametric data, respectively. Pearson's correlation coefficient was used to determine the significant association between variables. A p-value less than 0.05 was considered statistically significant.

Results

In this cross-sectional study of 70 newborns, there were 31 males (44%) and 39 females (56%). Mothers included in this study were between 20 and 38 years of age with a mean age of 26.59 years.

Maternal age and neonatal anthropometrical data (mean ± SD, range) are shown in Table 1. Of the term newborns, 37% (13/35) were males and 67% (22/35) were females, whereas in the preterm newborns, 51% (18/35) were males and 49% (17/35) were females. Term newborns weighed more and had higher anthropometry values (p<0.001) than preterm newborns.

Pearson's correlation values between cord blood glucose, insulin, and cortisol levels and insulin resistance indices in term and preterm newborns are shown in Tables 2 and 3. In the term newborns, plasma glucose was positively correlated with GIR (r=0.793, p<0.001) but negatively correlated with HOMA-IR (r=-0.481, p<0.01). Insulin was negatively correlated with QUICKI and GIR (r=-0.715, p<0.001 and r=-0.84, p<0.001, respectively) but positively with HOMA-IR (r=0.91, p<0.001). Cortisol was negatively correlated with HOMA-IR (r=-0.3, p<0.03) but was found to positively correlate with GIR (r=0.351, p<0.039).

In preterm newborns, plasma glucose showed positive correlations with GIR (r=0.47, p<0.004) and negative correlations with QUICKI (r=-0.39, p<0.02). Insulin levels showed a positive correlation with HOMA-IR (r=0.95, p<0.001) but a negative correlation with QUICKI and GIR (r=-0.406, p<0.01 and r=-0.72, p<0.001, respectively). Cortisol was found to positively correlate with glucose and GIR (r=0.376, p<0.02 and r=0.47, p<0.004, respectively) but negatively with insulin and HOMA-IR (r=-0.367, p<0.03 and r=-0.48, p<0.02, respectively).

Discussion

In this cross-sectional study, we estimated the levels of cord blood glucose, insulin, and cortisol in term and preterm newborns and the correlations between glucose homeostasis

Table 1. Descriptive data in term and preterm newborns and comparison of the two groups

	Term Mean ± SD (Minimum-Maximum)	Preterm Mean ± SD (Minimum-Maximum)	p-value
Mothers' age (years)	26.8±4.7 (20-38)	26.37±2.25 (21-31)	0.03*
Gestational age (weeks)	39±1.1 (37-41)	34.6±1.5 (29-36)	0.001*
Gender (Male/Female)	13/22	18/17	-
Birth weight (Kg)	2.96±0.33 (2.5-3.5)	2.1±0.23 (1.7-2.4)	0.001*
Crown-rump length (cm)	48.6±2.2 (44-53)	43.2±2 (41-49)	0.001*
Head circumference (cm)	33.1±1.5 (30-38)	32.45±1.2 (29-35)	0.038*
Chest circumference (cm)	35.8±1.2 (30.4-37.9)	30.6±0.73 (29-32)	0.001*
Waist circumference (cm)	27.9±1.2 (24-30.3)	24±0.63 (23-25.8)	0.001*
Mid arm circumference (cm)	9.8±0.75 (8-11.5)	8.1±0.27 (7.9-8.8)	0.5#
Insulin level (µIU/mL)	7.9±2.7 (3-16)	10.8±4.8 (4.8-21)	0.003*
Cortisol level (µg/dL)	11.88±5.78 (3-26)	8.9±4.66 (4.4-24)	0.021*
Glucose level (mg/dL)	83.6±17.4 (46-116)	70.4±15.8 (36-99)	0.002*
HOMA-IR	1.19±0.29 (0.9-1.49)	1.52±0.66 (0.78-2.28)	0.02*
QUICKI	0.35±0.01 (0.33-0.39)	0.36±0.01 (0.31-0.4)	0.07#
GIR**	10.2 [7.6, 16.6] (2.9-38.7)	7.1 [5.1, 10.8] (1.8-20)	0.056$

HOMA-IR: Homeostatic model assessment of insulin esistance, QUICKI: Quantitative insulin sensitivity check index, GIR: glucose insulin ratio, SD: standard deviation. **Median [Q_1, Q_3], $Mann-Whitney U test p>0.05, #Independent t-test p>0.05, *Independent t-test p<0.05

indices and insulin resistance markers. Glucose homeostasis indices in this study were derived from the estimated glucose, insulin, and cortisol levels. These levels were 36-116 mg/dL, 3-21 µIU/mL, and 3-26 µg/dL, respectively in the cord blood of the newborns included in the study. The glucose, insulin, and cortisol concentrations determined in our study population concur with previously reported levels which were 37-113 mg/dL, 3-21 µIU/mL, and 7-31.3 µg/dL, respectively for glucose, insulin, and cortisol (15,16,17).

In term newborns, mean glucose levels (83.6±17.4 mg/dL) were significantly higher than in preterm newborns (70.4±15.8 mg/dL), whereas insulin levels were found significantly lower in term newborns (7.9±2.7 µIU/mL) than in preterm newborns (10.8±4.8 µIU/mL). This confirms the role of insulin in glucose utilization indicating that glucose concentration decreases as the insulin level increases. In the current study, the higher insulin levels in preterm newborns than term newborns ratifies its higher requirement in preterm newborns for their growth and development (16).

Different values of cord blood cortisol level of term newborns have been reported. Gesteiro et al (15), Kırımi and Gül (17), and Sano et al (18) have reported cord blood values for term newborns as 4.4-10.4 µg/dL, 5.73-21.5 µg/dL, and 70-313 ng/mL, respectively (16). In this present study, mean cortisol level for preterm newborns was 8.9±4.66 µg/dL (4.4-24 µg/dL), a value lower than that for term newborns, i.e. 11.88±5.78 µg/dL (3-26 µg/dL). This may be attributed to the major regulatory action of cortisol in the final maturation of the fetus and in neonatal adaptation at birth. The fetal cortisol level remains low till 30 weeks of gestation and then progressively rises to reach 200 µg/mL near term (17,19). At the last stage of pregnancy, cortisol level increases in parallel to the development of fetus. However, the substantial direct effect of cortisol on birth weight is not yet established (17).

In preterm neonates, the adaptation process is very difficult to accomplish as they are at a higher risk of altered glucose homeostasis. They frequently develop hypoglycemia as a result of small substrate stores and immature enzyme systems (1). Insulin sensitivity is the ability of insulin to decrease plasma glucose levels by suppressing hepatic glucose formation and stimulating glucose utilization in skeletal muscle and adipose tissue, while insulin resistance is described as an impaired biological response to insulin (15).

The HOMA-IR, QUICKI, and GIR indices have rarely been tested in cord blood of newborns (20). In the present study, significantly increased insulin levels and HOMA-IR values were noted in the cord blood of preterm newborns as compared to term newborns. This shows that low birthweight newborns were at a higher risk of developing obesity and type 2 diabetes in later life considering the immature growth of β cells of pancreas in preterm newborns (21). In this study, no significant differences were found for QUICKI and GIR between term and preterm newborns despite the fact that preterm newborns had higher values. This may be due to the low prevalence of insulin resistance in neonates than older children (1).

In term newborns, glucose was found to negatively correlate with HOMA-IR but showed a positive correlation with GIR. This confirms the glucose utilization effect of insulin along with the added role of receptors activity in term newborns. Insulin had a strong positive correlation with HOMA-IR and a negative correlation with QUICKI and GIR. However, these associations were more significant in term than in preterm newborns. Bleicher et al (22) have shown significant correlations between cortisol and HOMA-IR in pediatric patients, indicating that cortisol contributes to insulin resistance. In contrast to Bleicher et al (22) we found significant negative correlation between cortisol and HOMA-IR and significant positive correlation between cortisol and GIR. In support of this finding, Adam et al (23) have also reported that cortisol has negative association with insulin secretion from the pancreas thus causing hyperglycemia and insulin resistance.

Our data shows a significantly higher correlation of cord blood cortisol with GIR in preterm newborns as compared to term newborns. It is known that low birth weight is partially responsible for hyperactivity of the hypothalamic–pituitary-adrenal axis which causes a state of functional hypercortisolism. Thus, increased cortisol levels and greater responsiveness of the hypothalamic–pituitary-adrenal axis may play an important role in the development of metabolic syndrome at both central and peripheral level in later life (16).

Table 2. Correlations of cord blood glucose, insulin, and cortisol levels with insulin resistance indices in term newborns

	Glucose		Insulin		Cortisol	
	r	p	r	p	r	p
HOMA-IR	**-0.481***	**0.01**	**0.91***	**0.001**	**-0.3***	**0.03**
QUICKI	0.073	0.67	-0.715*	0.001	0.15	0.39
GIR	0.793*	0.001	-0.84*	0.001	0.351*	0.039

HOMA-IR: Homeostatic model assessment of insulin esistance, QUICKI: Quantitative insulin sensitivity check index, GIR: glucose insulin ratio. *p<0.05 is statistically significant

Table 3. Correlations of cord blood glucose, insulin, and cortisol levels with insulin resistance indices in preterm newborns

	Glucose		Insulin		Cortisol	
	r	p	r	p	r	p
HOMA-IR	**-0.29**	**0.06**	**0.95***	**0.001**	**-0.48***	**0.02**
QUICKI	-0.39*	0.02	-0.406*	0.01	-0.24	0.15
GIR	0.47*	0.004	-0.72*	0.001	0.47*	0.004

HOMA-IR: Homeostatic model assessment of insulin esistance, QUICKI: Quantitative insulin sensitivity check index, GIR: glucose insulin ratio. *p<0.05 is statistically significant

The obtained levels of cord blood glucose, insulin, and cortisol in normal term infants (83.6±17.4 mg/dL, 7.9±2.7 µIU/mL, and 11.88±5.78 µg/dL, respectively) and in preterm infants (70.4±15.8 mg/dL, 10.8±4.8 µIU/mL, and 8.9±4.66 µg/dL, respectively) can be used as our own reference for further studies as there are no studies on cord blood normal levels in term and preterm newborns in India. Increased cord blood insulin level and HOMA-IR in preterm infants show a risk for developing insulin resistance in preterm newborns.

The foremost limitation to our study is that it reports cross-sectional data, which prevented us from drawing causal relationships. Longitudinal larger scale studies are required to validate our findings and show any incremental prognostic information about chances of developing diabetes and insulin resistance in term and preterm newborns.

In conclusion, we hope that the cord blood levels of glucose, serum insulin, and cortisol as well as the HOMA-IR, QUICKI, and GIR reported in this study may help other researchers to create reference ranges in term and preterm newborns. Higher insulin levels and HOMA-IR values in preterm newborns at birth supports the hypothesis that states which can lead to obesity, hyperinsulinemia, and insulin resistance in later life can have an intrauterine origin. Elucidation of the underlying mechanisms may offer an opportunity to influence body composition of the preterm newborns and therefore their susceptibility to future risk of developing diabetes and insulin resistance. The ever increasing incidence of diabetes in populations can be assessed with a new perspective at birth itself and also as an initiative for an early intervention.

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Ethics

Ethics Committee Approval: Kasturba Medical College, Mangalore, India and 18/9/2013, Informed Consent: It was taken.

Peer-review: External and Internal peer-reviewed.

Authorship Contributions

Concept: Afzal Ahmad, Design: Rukmini M. S., Data Collection or Processing: Afzal Ahmad, Charu Yadav and Ashish Agarwal, Analysis or Interpretation: Afzal Ahmad, Ashish Agarwal and Anupama Hegde, Literature Research: Afzal Ahmad, Rukmini M. S. and Poornima A. Manjrekar, Writing: Afzal Ahmad, Rukmini M. S..

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Original Article

Regulatory T Cells and Vitamin D Status in Children with Chronic Autoimmune Thyroiditis

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ABSTRACT

Objective: It is suggested that vitamin D is one of the factors that can regulate the function of Treg cells. In this study, the relationships between Treg cells and vitamin D levels was investigated in pediatric chronic autoimmune thyroiditis (CAT) patients.

Methods: Thirty-two children with CAT and 24 healthy subjects were studied. FOXP3 expressing $CD4^+CD25^{+high}$ $Foxp3^+$T cells were identified as Treg cells. At diagnosis, 25-hydroxycholecalciferol (25OHD3) levels were determined in all patients. FOXP3 expression was measured before and after vitamin D replacement therapy in patients having low levels of 25OHD3.

Results: In the CAT patients, Treg cell levels did not differ from the control group, while the frequency of vitamin D deficiency was higher and FOXP3 molecule expression was lower. FOXP3 molecule expression significantly increased in CAT patients having vitamin D deficiency who were given vitamin D replacement.

Conclusion: FOXP3 expression is decreased in pediatric CAT patients. This reduction seems to be associated with vitamin D levels. Vitamin D can play a role in enhancing natural Treg cell functions.

Keywords: Treg cells, chronic autoimmune thyroiditis, vitamin D

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

It is suggested that vitamin D is one of the factors that can regulate the functions of Treg cells.

WHAT THIS STUDY ADDS?

In the pediatric patients with chronic autoimmune thyroiditis, reduction of FoxP3 expression seems to be associated with vitamin D levels. Vitamin D can play a role in enhancing natural Treg cells functions.

Introduction

Chronic autoimmune thyroiditis (CAT) is the most common form of thyroiditis encountered in childhood and in the adolescent period (1). Similar to other autoimmune diseases, there are complex interactions between genetic susceptibility and environmental factors in the pathogenesis of CAT development. The loss of immune tolerance to self-thyroid antigens leads to autoimmune

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thyroid diseases (AITDs) (2). CAT is a T cell-mediated disease which is characterized by an activation of self-reactive CD4+ T lymphocytes, heavy T-cell infiltration in the thyroid gland, and progressive destruction of thyrocytes (3).

T cells have several subpopulations such as CD4+ helper, CD8+ cytotoxic, and regulatory T cells (Tregs) (4). Tregs are a specialized subset of T cells that are co-expressed as CD4+ and CD25+, which have a central role for immune tolerance by downregulating the inflammatory response against self-antigens (5,6,7). These cells exert their regulatory activity on CD4+ T cells, CD8+ T cells, B cells, and dendritic cells (4). One of the important characteristics of Tregs is the expression of the forkhead/winged helix transcription factor FOXP3 molecules that serve as keys in the maintenance of peripheral tolerance and in controlling the immune response (2). FOXP3 is a key gene in the development of Treg cells and it commits naive T cells to become Treg cells (4). Recent studies suggest that a decrease in FOXP3+Treg cells number and/or function is often associated with autoimmunity (8,9). While there is a growing number of studies on Treg cells in AITD in recent years (8,9), studies on autoimmune thyroiditis in humans are still limited. The exact role of Treg cells in the pathogenesis of CAT has not yet been fully recognized.

In recent years, increasing attention has also been drawn to the relationship between autoimmune diseases and vitamin D. Many cells of the immunological system, including T and B lymphocytes express vitamin D receptor and vitamin-D activating enzyme CYP27A1. Low levels of vitamin D were reported in patients with autoimmune diseases such as type 1 diabetes, lupus erythematosus, rheumatoid arthritis, and multiple sclerosis (2). Vitamin D is accepted as an immunomodulatory molecule. Some *in vitro* studies showed that vitamin D inhibits T cell proliferation. Vitamin D also appears to influence Treg cells differentiation and activity (10). Vitamin D stimulates the differentiation and activation of CD4+ lymphocytes, inhibits the differentiation of monocytes and dendritic cells and reduces the production of proinflammatory cytokines by Th1 cells (11). In addition, vitamin D may influence autoimmune disease risk and severity by dampening pathogenic Th17 cell IL-17 synthesis, amplifying a Th1–Tr1 switch (12).

Along with publications supporting the beneficial effect of vitamin D supplementation on autoimmunity, some studies could not demonstrate this effect (13,14). The situation with respect to CAT is not yet precisely clear.

As far as we know, the effect of vitamin D supplementation on Treg cells has not been demonstrated in children and adolescents with AITDs. In this study, aiming to contribute to clarification of the etiology of AITDs, we evaluated vitamin D status in CAT patients and in healthy children and adolescents in relation to the function of FOXP3+ Treg cells. We also investigated the possible changes in FOXP3 expression with vitamin D supplementation in vitamin D-deficient CAT cases.

Methods

The protocol for the study was approved by the Ethics Committee of Ankara University. Informed consent was obtained from all individual participants included in the study and their parents. The study was conducted between April 2013 and May 2014. Thirty-two children and adolescents with a diagnosis of CAT and 24 healthy sex- and age-matched subjects as a control group were enrolled to the study. The subjects in the control group were euthyroid and their were negative.

Inclusion criteria for both the CAT and control groups were as follows: 1) no experienced acute infection or any other illness during the 2 months period to the study; 2) absence of any other disease (hepatic, renal, immunological, etc.); 3) no medications including immunoactive drugs.

Clinical examination and assessment of pubertal status were performed in all subjects. The diagnosis of CAT was made by using conventional clinical, laboratory and ultrasonographic findings.

Blood samples were collected in the morning between 8:30 and 9:30 a.m. for serum levels of free triiodothyronine (fT_3), free thyroxine (fT_4), thyroid-stimulating hormone (TSH), antithyroid antibodies, 25-hydroxycholecalciferol (25OHD3) and for measurement of Tregs with FOXP3 expression. Determinations of TSH, anti-thyroid peroxidase antibody (anti-TPO), anti-thyroglobulin antibody (TgAb), fT_4, and fT_3 levels were performed by electro-chemiluminescence immunoassay (ECLIA) using Roche® Elecsys reagent. Normal values ranged between 7 and 16 pmol/L for fT_4, 3.8 and 6 pmol/L for fT_3, and 0.34 and 5.6 mIU/mL for TSH.

The negative values for antithyroid antibodies were 0-4 IU/mL for anti-TPO and 0-9 IU/mL for anti-TG. The 25(OH)D levels were determined chromatographically using the isocratic high-performance liquid chromatography system.

Thyroid ultrasonography (US) was performed by an experienced radiologist. Subjects were examined with 7 MHz linear and an SSA 770 Aplio scanner (Toshiba Medical Systems Co, Ltd, Tokyo, Japan).

Immunostaining and Flow Cytometric Analysis

The frequency and precise number of Tregs were determined by three-color flow cytometry analysis performed on fresh whole blood collected in EDTA anticoagulant tubes. The blood cells were stained with antiCD4-PC5 (Beckman Coulter, Marseille, France), anti-CD25FITC (Beckman Coulter, Marseille, France), and intracellular anti-FOXP3-PE (eBioscience, San Diego, CA). Isotype-matched antibodies were used as negative controls. The blood samples were incubated at 4 °C for 45 minutes. After washing the cells twice with permeabilization buffer, fluorochrome-conjugated anti-human FOXP3 antibody (eBioscience, Cat. 12-4776. Clone PCH101) or isotype control were added. They were incubated at 4 °C for at least 30 minutes. Staining with mAb-anti-FOXP3 (antihuman

FOXP3, eBioscience) was achieved according to the protocol recommended by the manufacturer with staining buffer set (eBioscience).

Three subsets of CD4+ T cells were defined according to CD25 staining: $CD25^{-}$, $CD25^{low}$, and $CD25^{high}$. Cells expressing $CD25^{high}$ were chosen and gated for the detection of FOXP3+ T cells (Figures 1 and 2a-2b). Three-color flow cytometry with Kaluza Software version was used for analysis by using NAVIOS®, Beckman Coulter, Miami. One single laboratory technician performed the flow cytometric analysis of Treg cells to avoid interindividual differences in technique.

Serum 25OHD3 levels below 20 ng/mL were considered as vitamin D deficient (15). Patients with low serum 25OHD3 levels were started on oral vitamin D3 (400 U/day) treatment. 25OHD3 levels were checked monthly. At least one month after the normalization of vitamin D levels, the percentage of Treg cells and FOXP3 expression were reanalyzed.

Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) for Windows 11.5 was used for statistical analysis. Student's t-test, Fisher's exact test, and Mann-Whitney U test were used to assess the differences between the groups. Wilcoxon signed ranks test was performed to compare $CD4^{+}CD25^{+high}$ T cell levels before and during treatment in the vitamin D-deficient patients. Statistical significance was considered when $p < 0.05$.

Results

In this study, 32 newly diagnosed CAT patients (aged 5 to 18.4 years) and 24 subjects as a control group were evaluated. The CAT (28 female, 4 male) and control groups (18 female, 6 male) were essentially similar with respect to age, height, and weight SDS. Although 7 (21.8%) of the patients in the study group had hypothyroidism, there was no statistically significant difference between the groups according to overall thyroid hormone levels (Table 1). Four of the hypothyroid subjects had compensated, while 3 had uncompensated hypothyroidism. There was also no significant correlation between thyroid hormone levels and the percentages of Treg cells in the CAT patients.

In the study group, the percentage of $CD4^{+}CD25^{+}FOXP3^{+high}$ T cells (Treg cells) did not differ from that in the control group, while FOXP3 molecule expression was low (p=0.01). Vitamin D levels were also lower in the CAT group than in the control group (16.02 vs. 21.91, p=0.045). In the study group, there was a correlation between FOXP3 molecule expression and serum vitamin D levels (r=0.38, p=0.042).

The frequency of 25OHD3 D deficiency was higher in the study group as compared to the controls (68.7%-22 of 32 subjects) vs. 41.6%-10 of 24 subjects), respectively (p=0.04). Only one patient had hypothyroidism and normal vitamin D level on admission. Vitamin D levels were similar in the hypothyroid and euthyroid subjects (12.41 vs. 18.7 mcg/L, p=0.08).

Treg cells percentage and their FOXP3 molecule expression were reevaluated after replacement therapy in CAT patients with vitamin D deficiency when they achieved euthyroid status. Twelve CAT patients with low vitamin D levels were analyzed after vitamin D replacement. Vitamin D replacement was continued for 2 to 5 months (mean: 3 months).

The percentage of Treg cells did not change in the CAT patients with vitamin D deficiency who were given vitamin D replacement. On the other hand, FOXP3 molecule expression increased significantly (p=0.013). After vitamin D replacement, a statistically significant decrease in the level of thyroid antibodies was observed in CAT patients (Table 2).

Figure 1 shows gating strategy for frequency of Treg cells in the study population. FOXP3 expression of a patient with a low level of vitamin D at diagnosis and after vitamin D replacement is shown at Figure 2a-2b.

Discussion

Treg cells, by modulating potentially self-reactive T cells through secreting cytokines or by direct cell contact dependent mechanisms, play a crucial role in immune tolerance (7). The role of Treg cells in human autoimmune disease and in the pathogenesis of CAT is still not clear and under evaluation (16). It is suggested that patients with autoimmune diseases may have dysfunction or depletion of Treg cells (7).

In this study, the percentage of Treg cells and the expression of FOXP3 in children with a diagnosis of CAT was evaluated. The percentage of Treg cells in the CAT patients was found to be essentially similar to that of healthy children. However, there was a statistically significant reduction of FOXP3 expression in the CAT group when compared to the control group.

Table 1. Characteristics of the subjects in the patient and control groups

	CAT patients	Control group	p-values
Age (years)	13.24±3.74	12.76±3.36	0.36
n (Female/Male)	28/4	18/6	-
Height SDS (z score)	0.084±1.24	0.087±1.58	0.49
BMI (kg/m^2)	22.01±4.08	21.14±4.34	0.31
TSH (mIU/mL)	12.63±29.45	2.6±1.47	0.08
fT_4 (pmol/L)	10.45±2.79	12.2±6.05	0.12
25OHD3 (mcg/L)	16.02±9.84	21.91±7.68	0.045
FOXP3 molecule expression (%)	70.46±16.06	82.1±14.88	0.01
Treg cells (%)	3.6 ±1.5	3.8 ±1.5	0.25

CAT: chronic autoimmune thyroiditis, SDS: standard deviation score, BMI: body mass index, TSH: thyroid-stimulating hormone, fT_4: free thyroxine, 25OHD3: 25-hydroxycholecalciferol

In a previous study, Ban et al (17) tested the *FOXP3* gene locus for association with AITD in two large cohorts. They found no association between FOXP3 polymorphisms and AITD in the Japanese cohort, while there was a significant association in the Caucasian cohort. In another study, it was shown that the upregulation of Treg cells can suppress experimental thyroiditis (6).

The number of Treg cells was reported as adequate, increased, or decreased in AITD in several studies (8,9). Marazuela et al (8) showed an increase in the number of CD4+ CD25 T cells with molecular defect (a disturbed expression of IL-10, transforming growth factor-beta, genes for transcription factors FOXP3, STAT1, STAT3, and genes critical to Treg cells) in patients with AITD. They demonstrated that similar cells infiltrated into the thyroid tissue in CAT patients. These same authors concluded that the suppressive function of Tregs in peripheral blood was incomplete in these patients. An important study conducted on children with newly diagnosed AITD demonstrated a statistically significant reduction in the percentage of Tregs with the phenotype $CD4^{+}CD25^{high}$ and CD4+FOXP3 in children with AITD when compared to healthy children (9). In our study, patients with CAT showed a low FOXP3 expression compared to the control group, while Treg percentage was not different. The functional defect of Treg cells, similar to the low FOXP3 expression, could be specific for development of CAT. Either decreased number or impaired function of Treg cells may lead to the development of AITDs. Beside, increasing the FOXP3 level, upregulating the Treg cell functions may be an option for decreasing autoimmune responses in CAT patients.

Another aspect of our study was to evaluate vitamin D levels of the subjects and search for any relationship with Treg cells function, since Treg cells differentiation and activity can be influenced by vitamin D. Vitamin D has anti-inflammatory and immunomodulatory effects. In animal models, it was shown that administration of 1,25-dihydroxyvitamin D3 (or its analogs) arrests the immunological progression, thus preventing the clinical onset of autoimmune diseases (18). Indeed, vitamin D agonists have regulatory effects on the activation and differentiation of T-cells. It is also hypothesized that vitamin D deficiency can act as an environmental trigger that increases the occurrence of AITD (19).

Table 2. FOXP3 molecule expression and percentage of Treg cells in patients with vitamin D deficiency before and after vitamin D replacement

	Before vitamin D replacement	After vitamin D replacement	p-values
FOXP3 molecule expression (%)	70.7±15.38	86.2±6.72	0.015
Treg cells (%)	3.9±1.31	4.5±1.5	0.23
Anti-TPO (U/L)	128±38	44±19	0.02

Anti-TPO: anti-thyroid peroxidase antibody

Until now, the mechanisms underlining the role of vitamin D in thyroid autoimmunity are not completely understood. The relationships between thyroid autoimmunity and vitamin D have not been studied extensively. There are also conflicting results about the association of AITD with vitamin D levels. Although some papers do not confirm the connection between vitamin D levels and AITD, lower levels of vitamin D have been reported in several autoimmune diseases including Hashimoto's thyroiditis and Graves' disease (13,20,21,22).

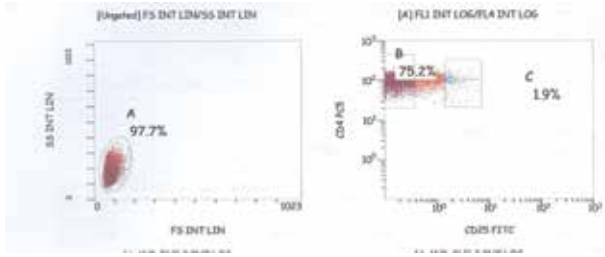

Figure 1. Gating strategy for frequency of Treg cells in the study population. Three color flow cytometry was performed in whole blood. Three subsets of $CD4^{+}$T cells were defined according to CD25 staining cells expressing CD25 high were chosen and gated for the detection of FOXP3+T cells

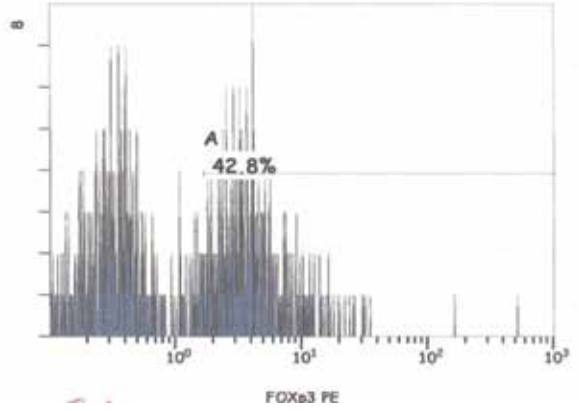

Figure 2a. FOXP3 expression of a patient with a low level of vitamin D at diagnosis

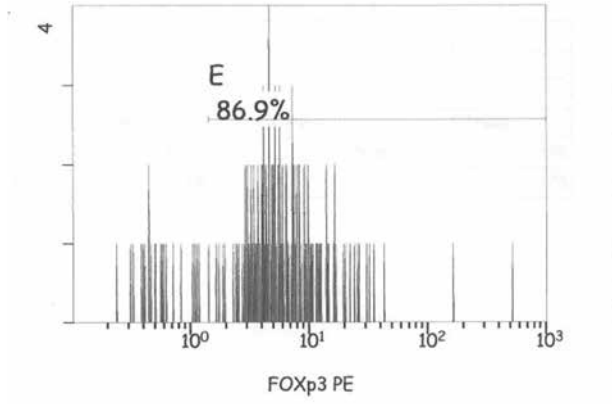

Figure 2b. FOXP3 expression of a patient after vitamin D replacement

Evliyaoğlu et al (23) have investigated vitamin D status in children and adolescents with CAT, and they found that CAT was observed 2.28 times more frequently in individuals with 25(OH)D3 levels <20 ng/mL. Camurdan et al (22) reported lower levels of vitamin D and higher vitamin D deficiency rates in children with Hashimoto's thyroiditis when compared to the control group (22). In contrast, D'Aurizio et al (14) and Effraimidis et al (13) demonstrated that vitamin D levels were not lower in AITD patients than in controls. Effraimidis et al (13) carried a case-control study in subjects with normal TSH levels and no thyroid antibodies. During follow-up, cases with TPO antibody and controls with no TPO antibodies did not show any differences in their vitamin D levels. In the present study, we found a lower level of vitamin D in the study group than in the control group, also the frequency of vitamin D deficiency was higher in the study group on admission.

The cause of inconclusive results of low vitamin D level and AITD is not completely understood. Heterogeneous characteristics of study populations, levels of thyroid dysfunction, seasonal variability of blood sampling, the duration of disease, characteristics of the control groups, incidence of vitamin D deficiency in the population could be factors affecting vitamin D levels.

In our vitamin D-deficient CAT patients, after vitamin D replacement had been given, Treg cells percentage and FOXP3 expression were reanalyzed. We showed that vitamin D replacement could induce FOXP3 expression in children with CAT. With treatment, anti-TPO antibody titers showed a decrease. Although the number of cases is limited, this result gives a clue that vitamin D replacement can affect Treg cell function by increasing FOXP3 expression. For vitamin D treatment, we used the physiological replacement dose of 400 U/day. We do not know if higher doses of vitamin D would lead to more prominent changes in the percentage or function of Treg cells.

Although the number of patients is limited in our study, our results indicate an increased prevalence of vitamin D deficiency in patients with CAT. We based our findings on normal levels of vitamin D with respect to bone metabolism, even though these levels might vary for different organs or systems. In other words, adequacy of vitamin D levels for the immune system could differ from that for the skeletal system.

Factors such as some genetic and/or environmental changes could possibly affect FOXP3 expression. We do not know which of these factors are more effective in inducing the development of AITD. Hypothyroidism or LT4 treatment are additional factors which should have be taken into consideration. Actually, it has been suggested that LT4 therapy in hypothyroid patients can affect Treg cells functions (9). It has been shown that in hypothyroid patients, LT4 therapy leads to a decrease in IL-12, and it was suggested that a cytokine could be responsible for Th1 cell differentiation (24). In our study, there was no difference in serum vitamin D levels between hypothyroid and euthyroid patients in the CAT group. FOXP3 expression showed a similar increase in both euthyroid and hypothyroid CAT patients after replacement with vitamin D. These findings might confirm the immunomodulatory effects of vitamin D replacement. The decrease in anti-TPO titer in patients with vitamin D replacement is noteworthy. This finding might be reflecting the improvement of Treg cell functions of the patients after vitamin D replacement.

To conclude, in pediatric patients with CAT, FOXP3 molecule expression is decreased and this reduction appears to be associated with vitamin D levels. In patients requiring vitamin D, after replacement in physiological doses, the expression of FOXP3 molecules showed an increase. This result suggests that vitamin D can play a role in enhancing natural Treg cells functions.

Ethics

Ethics Committee Approval: Ankara University Ethic Committe Approval Number 152-47-88, Informed Consent: It was taken.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Zeynep Şıklar, Design: Zeynep Şıklar, Data Collection or Processing: Zeynep Şıklar, Bülent Hacıhamdioğlu, Analysis or Interpretation: Zeynep Şıklar, Merih Berberoğlu, Deniz Karataş, Aydan İkincioğulları, Figen Doğu, Literature Research: Zeynep Şıklar, Writing: Zeynep Şıklar, Merih Berberoğlu.

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Original Article

Urinary Netrin-1: A New Biomarker for the Early Diagnosis of Renal Damage in Obese Children

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ABSTRACT

Objective: Urinary netrin-1 is a new marker to demonstrate early tubular damage. The aim of this study was to determine whether urinary netrin-1 is increased in obese children.

Methods: A total of 68 normoalbuminuric and normotensive obese patients and 65 controls were included in the study. Urine samples were collected for assessment of urinary phosphorus, sodium, potassium, creatinine, albumin, and netrin-1. Blood samples were collected for measurements of fasting glucose, insulin, lipid, phosphorus, sodium, potassium, and creatinine levels. Homeostatic model assessment insulin resistance index was calculated.

Results: Gender and age were similar between obese and control groups (12.01±3.03 vs. 11.7±3.2 years, p=0.568 and 33 vs. 35 girls, p=0.543, respectively). Obese patients had significantly higher netrin-1 excretion than the controls (841.68±673.17 vs. 228.94±137.25 pg/mg creatinine, p=0.000). Urinary netrin-1 level was significantly higher in obese subjects with insulin resistance compared to those without insulin resistance (1142±1181 vs. 604.9±589.91 pg/mg creatinine, p=0.001).

Conclusion: In normotensive and normoalbuminuric obese children, urinary netrin-1 level can increase before onset of albuminuria. Urinary netrin-1 excretion appears to be affected predominantly by insulin resistance and hyperinsulinemia. Urinary netrin-1 may be a new biomarker for determining early tubular injury in obese children.

Keywords: Obesity, insulin resistance, tubular dysfunction, netrin-1, children

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Netrin-1 is a promising early biomarker of tubular kidney injury. The impact of obesity on chronic kidney disease has been well demonstrated.

WHAT THIS STUDY ADDS?

Netrin-1 is significantly elevated in obese patients without microalbuminuria. Netrin-1 was higher in obese with insulin resistance than without insulin resistance.

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Introduction

The impact of obesity on chronic kidney disease (CKD) has been well demonstrated (1,2). The diagnostic test currently used for CKD in clinical practice is increased albumin excretion rates. However, this is a sign of early glomerular damage rather than a marker for susceptibility to it. Also studies assert that tubulointerstitial injury may precede the appearance of glomerulopathy in diabetic nephropathy (3,4). Therefore, identification and validation of tubular injury biomarkers for early diagnosis of kidney injury come into prominence.

The netrin-1 is a conserved family of laminin-related proteins that were originally identified as axonal guidance cues (5). Many studies have demonstrated netrin-1 expression outside the nervous system, including the kidneys (6). Using both *in vitro* and *in vivo* systems, netrin-1 was shown to play a role in promoting angiogenesis, cell migration, tissue morphogenesis, and in regulation of inflammation (7,8). Netrin-1 protein expression has been localized to endothelial cells in the normal kidney, and very little protein expression is seen from tubular epithelial cells (9). After injury, netrin-1 protein expression appears in proximal tubular epithelial cells but, at the same time, the expression in vascular endothelial cells is down regulated. Since netrin-1 is a secreted protein in urine, this led to the discovery of netrin-1 as an early diagnostic biomarker of kidney injury (8,9). It was found that netrin-1 is a secreted protein highly induced after acute and chronic kidney injury and excreted in urine in both mice and humans (10,11).

Reports in the literature show that tubular changes such as hypertrophy, reduced ion transport, and thickening of the basement membrane are already apparent before the onset of proteinuria in early diabetic nephropathy (3,4). In a recent study, urinary netrin-1 levels were significantly increased in normoalbuminuric diabetic adult patients when compared to healthy controls and still further elevated in patients with microalbuminuria and overt nephropathy (12). We have hypothesized that urinary netrin-1 excretion was higher in obese children than healthy children. We therefore compared the netrin-1 levels between normoalbuminuric/normotensive obese children and controls. Furthermore, we examined the risk factors affecting the level of urinary netrin-1.

Methods

This cross-sectional study examined two groups (obese patients and healthy controls) attending the pediatric endocrinology outpatient clinic of our hospital. All patients provided written informed consent, and our ethics committee approved the study protocol. Financial support was received from the Institute's Epidemiological Committee.

Obesity was defined as a body mass index (BMI) ≥95th percentile for age and sex. Normal weight was defined as BMI <85th percentile for age and sex (13). Normotension was defined as a systolic or diastolic blood pressure <90th percentile according to age, sex, and height on at least three occasions. Hypertension was defined as systolic or diastolic blood pressure ≥95th percentile according to age, sex, and height on at least three occasions (14). We used the United States National Heart, Lung, and Blood Institute (NHLBI) definition for dyslipidemia, abnormal values being >95th percentile, except for high density lipoprotein, for which an abnormal value is less than 10th percentile (15). Insulin resistance was defined according to the homeostatic model assessment of insulin resistance (HOMA-IR). HOMA-IR was estimated from fasting plasma measurements [insulin (μU/L) X glucose (mg/dL)/405]. Insulin resistance criteria were HOMA-IR >2.67 for prepubertal boys, >2.22 for prepubertal girls, >5.22 for adolescent boys, and >3.82 for adolescent girls (16). Microalbuminuria was defined according to the urine albumin-to-creatinine ratio. A value of >30 mg/g for creatinine suggests moderately increased albumin excretion (17).

Each child underwent a complete physical examination and anthropometric measurements, including pubertal staging according to the Tanner criteria (18,19). Height and weight were measured in postabsorptive conditions and with an empty bladder. Height was measured to the nearest 0.5 cm on a standard height board, and weight was determined to the nearest 0.1 kg on a standard physician's beam scale with the subject dressed only in light underwear and no shoes. To compare BMI across different ages and between genders, BMI standard deviation score (SDS) was calculated.

Patients with the following were excluded from the control group: obesity; chronic disease; family history of stroke, diabetes or dyslipidemia, use of any medications during the study period or in the preceding 6 weeks, and existing or previous infections in the preceding 6 weeks according to the patients' clinical history and physical examination. Participants in the study were selected from among healthy children who presented to our pediatric outpatient clinic for evaluation of fitness for sportive activities.

The inclusion criteria for the study group consisted of being obese, normoalbuminuric, and normotensive. Exclusion criteria were hypertension, diabetes mellitus, microalbuminuria, syndromic obesity (Prader-Willi, Laurence-Moon Biedl syndrome, etc.), endocrine disease such as Cushing's syndrome or hypothyroidism, systemic disease including liver disease, malignancy, as well as existing or previous infection and drug use.

Fresh first morning urine samples were collected for determination of urinary phosphorus, sodium, potassium, creatinine, and netrin-1 levels. Normoalbuminuria was confirmed by two different first morning urine samples. Blood samples were also taken for determination of fasting glucose, insulin, total cholesterol, low-density lipoprotein (LDL), triglyceride, phosphorus, sodium, potassium, and creatinine levels. All determinations were done within 4 hours of sample collection,

except for netrin-1, where urine samples were centrifuged (3,000 rpm for 20 min) and stored at -80 °C until measurement. Estimated glomerular filtration rate (eGFR) was calculated according to the Schwartz formula (20). The fractional excretion of sodium (FE_{Na}), fractional excretion of potassium (FE_K), and tubular phosphate reabsorption (TPR) rate were calculated using standard formulas.

Serum and urinary phosphorus, creatinine, total cholesterol, LDL, and triglycerides were measured by the colorimetric method; serum and urinary sodium and potassium were determined by indirect method in ISE module with Abbott Architect autoanalyzer C16000 (Illinois, USA) using home-made reagents. Serum creatinine was measured using the compensated Jaffe method. Urinary microalbumin was estimated using the nephelometric method with Siemens Nephelometry analyzer (Erlangen, Germany).

Urinary netrin-1 was measured using the enzyme-linked immunosorbent assay (ELISA) method (Sunred, Shanghai, China). Intra-assay and inter-assay precision were <9% and <11%, respectively. All assays were performed in duplicate. Urinary netrin-1 excretion is expressed in picograms (pg) per mg of creatinine.

All statistical calculations were performed using SPSS for Windows 15.0 (SPSS, Chicago, IL, USA). Comparison between two groups was performed using Mann-Whitney U-test for non-normally distributed parameters and student's t-test for parameters showing normal distribution. Comparison between subgroups was performed with Kruskal-Wallis tests. The means in more than two groups were compared using one-way ANOVA. Where the p-value was significant, pairwise comparisons were done with post-hoc Bonferroni test. Statistical significance was accepted as $p<0.05$. The parameters are expressed as mean values and standard deviations. Spearman correlation coefficient was used for correlation analysis. After adjustment for age and gender, we assessed the associations between netrin-1 and other relevant parameters by partial correlation analysis.

Results

A total of 62 obese patients and 64 control subjects participated in this study. The demographic characteristics and biochemical parameters of the two groups are shown in Table 1. There were no differences between the groups for age, gender, serum creatinine, eGFR, urine albumin-to-creatinine ratio ($p>0.05$). However, obese patients had significantly higher netrin-1 excretion when compared to controls [477.6 (240.3-864.5) vs. 240.9 (123.5-450.2) pg/mg creatinine, respectively, p=0.00064].

In the obese group, 11 subjects (17.7%) had dyslipidemia. When we compared the dyslipidemic and normolipidemic subjects, there were no differences in age, gender, BMI SDS, HOMA-IR, urine albumin-to-creatinine ratio, eGFR, FE_{Na}, FE_K, TPR, and urinary netrin-1.

We divided the obese subjects according to insulin resistance (Table 2). There were no differences between patients with and without insulin resistance for age, gender, BMI SDS, eGFR, urine albumin-to-creatinine ratio, FE_{Na}, FE_K, TPR, total cholesterol, and LDL. However, urinary netrin-1 was significantly higher in patients with insulin resistance than patients without insulin resistance [645.6 (480.4-1012.5) vs. 431.3 (207.2-682.5) pg/mg creatinine, p=0.005].

In order to evaluate the factors associated with urinary netrin-1, we performed correlation analyses (Table 3). There was no significant correlation between urinary netrin-1 and age, gender, BMI SDS, eGFR, urine albumin-to-creatinine ratio, FE_{Na}, FE_K, TPR, total cholesterol, and LDL. There were positive correlations between urinary netrin-1 and fasting

Table 1. The demographics and biochemical parameters of the obese and control groups

	Obese group (n=62)	Controls (n=64)	p-value
Age (years)	12.01±3.03	11.7±3.2	0.568
Gender (F/M)	30/32	33/31	0.543
BMI (kg/m^2)	29.28±5.83	18.31±3.87	0.000
BMI SDS	2.17±0.45	0.4±0.56	0.000
Serum creatinine (mg/dL)	0.72±0.12	0.73±0.13	0.649
eGFR	127.53±16.37	129.05±18.59	0.693
Urine albumin-to-creatinine ratio	16.38±5.58	14.74±4.17	0.134
FE_{Na} (%)	0.49±0.31	0.35±0.2	0.086
FE_K (%)	3.4±1.7	2.7±0.33	0.336
TPR (%)	96.86±3.15	95.48±2.58	0.270
Fasting Insulin (µU/L)	17.65±10.78	-	-
Fasting glucose (mg/dL)	92.5±12.4	-	-
HOMA-IR	4.07±2.71	-	-
Total cholesterol (mg/dL)	173.75±29.38	-	-
LDL-cholesterol (mg/dL)	96.68±19.56	-	-
Triglyceride (mg/dL)	104.5±24.6	-	-
Netrin-1 (pg/mg creatinine)	477.6 (240.3-864.5)	240.9 (123.5-450.2)	0.00064

Comparison between the two groups was performed using student's t-test for normally distributed parameters. The data are presented as means ± standard deviation. Comparison between the groups for netrin-1 levels was performed using Mann-Whitney U-test. The data are presented as median values (25th and 75th percentiles) ($p<0.05$). F: female, M: male, BMI: body mass index, SDS: standard deviation score, eGFR: estimated glomerular filtration rate, FE_{Na}: fractional sodium excretion, FE_K: fractional potassium excretion, TPR: tubular phosphorus reabsorption rate, HOMA-IR: homeostatic model assessment of insulin resistance, LDL: low-density lipoprotein

Table 2. Comparison of patients with insulin resistance with those without insulin resistance and controls

Parameters	Patients with insulin resistance (n=28)	Patients without insulin resistance (n=34)	Controls (n=64)	p	p*	p**	p***
Age (years)	11.77±3.21	12.22±2.89	11.53±3.19	0.578	0.645	1.000	0.888
Gender (F/M)	16/12	14/20	33/31	0.211	0.094	1.000	0.516
BMI (kg/m^2)	30.17±6.1	28.54±5.57	18.31±3.87	-	0.236	-	-
BMI SDS	2.28±0.44	2.07±0.45	0.4±0.56	-	0.106	-	-
Serum creatinine (mg/dL)	0.72±0.12	0.71±0.13	0.73±0.13	-	0.906	-	-
eGFR	128.29±16.18	127.44±15.93	129.05±18.59	-	0.673	-	-
Urine albumin-to-creatinine ratio	17.63±4.42	15.24±6.35	14.74±4.17	0.100	0.373	0.102	1.000
FE_{Na} (%)	0.38±0.3	0.56±0.31	0.35±0.2	0.092	0.168	1.000	0.102
FE_K (%)	3.83±2.25	2.85±0.76	2.7±0.33	0.419	0.724	0.640	1.000
TPR (%)	96.66±3.41	97.03±3.02	95.48±2.58	0.537	0.806	1.000	0.828
Total cholesterol (mg/dL)	165.03±26.21	181.36±31.05	-	-	0.058	-	-
LDL (mg/dL)	90.61±17.84	101.89±20.4	-	-	0.051	-	-
Netrin-1 (pg/mg creatinine)	645.6 (480.4-1012.5)	431.3 (207.2-682.5)	240.9 (123.5-450.2)	0.000	0.005	0.000	0.000

Comparison between the two groups was performed using student's t-test for normally distributed parameters. The data are presented as means ± standard deviation. Comparison between the groups for netrin-1 levels was performed using Mann-Whitney U-test. The data are presented as median values (25th and 75th percentiles) ($p<0.05$). F: female, M: male, BMI: body mass index, SDS: standard deviation score, eGFR: estimated glomerular filtration rate, FE_{Na}: fractional sodium excretion, FE_K: fractional potassium excretion, TPR: tubular phosphorus reabsorption rate, HOMA-IR: homeostatic model assessment of insulin resistance, LDL: low-density lipoprotein

Table 3. Factors associated with urinary netrin-1 in the obese group

Features	Correlation coefficient	p-value
Age (years)	0.035	0.781
Gender (F/M)	0.058	0.639
BMI (kg/m^2)	0.152	0.227
BMI SDS	0.130	0.302
Serum creatinine (mg/dL)	-0.055	0.690
eGFR	-0.083	0.523
Urine albumin-to-creatinine ratio	0.228	0.063
FE_{Na} (%)	-0.251	0.286
FE_K (%)	0.370	0.414
TPR (%)	-0.287	0.124
Fasting insulin (µU/L)	0.279	0.022
Fasting glucose (mg/dL)	0.433	0.000
HOMA-IR	0.345	0.004
Total cholesterol (mg/dL)	-0.134	0.307
LDL (mg/dL)	-0.069	0.599

eGFR: estimated glomerular filtration rate, FE_{Na}: fractional sodium excretion, FE_K: fractional potassium excretion, TPR: tubular phosphorus reabsorption rate, HOMA-IR: homeostatic model assessment of insulin resistance, LDL: low-density lipoprotein ($p<0.05$), F: female, M: male, BMI: body mass index, SDS: standard deviation score

insulin, glucose, and HOMA-IR (r=0.279, p=0.022 vs. r=0.433, p=0.000 vs. r=0.345, p=0.004, respectively). Partial correlation analysis was performed considering age and gender (Table 4). Even after considering age and gender, the results did not change.

Discussion

Baseline BMI has been suggested as an independent predictor of CKD progression (2). Metabolic syndrome, a major consequence of obesity, also seems to be an independent risk factor for end-stage renal disease (ESRD) (1). For this reason, investigations for a diagnostic test to detect early renal damage in clinical practice have increased. This present study has demonstrated that urinary netrin-1, which seems to be affected by tubular injury, is significantly elevated in obese patients without microalbuminuria when compared to controls. These results may suggest that tubular changes may already be apparent even before the glomerular injury process has begun in obese children.

Microalbuminuria currently used for CKD has significant limitations. The presence of microalbuminuria by itself may not be an adequate indicator for disease progression because of the observation that a number of type 1 diabetes patients revert to normoalbuminuria without treatment (21). Microalbuminuria is suggested to be a sign of early glomerular damage. The occurrence of microalbuminuria in type 1 diabetes can already

Table 4. Partial correlation analysis between urinary netrin-1 and fasting insulin, fasting glucose, and homeostatic model assessment of insulin resistance, controlling for age and gender

Parameters	Controlling for age Netrin-1 (pg/mg creatinine)		Controlling for gender Netrin-1 (pg/mg creatinine)	
	r	p	r	p
Fasting insulin (μU/L)	0.290	0.018	0.291	0.018
Fasting glucose (mg/dL)	0.435	0.000	0.445	0.000
HOMA-IR	0.357	0.003	0.361	0.003

HOMA-IR: Homeostatic model assessment of insulin resistance, p<0.05

be associated with diabetic nephropathy lesions comparable to the ones found in overt diabetic nephropathy (21). Therefore, identification and validation of biomarkers for early diagnosis of kidney injury may help develop effective treatment for kidney disease. For this purpose, tubular injury biomarkers such as kidney injury molecule-1 (KIM-1), N-acetyl-β-D-glucosaminidase (NAG), and neutrophil gelatinase-associated lipocalin (NGAL) were investigated in patients with obesity (22). Our study revealed that obese patients had higher urinary NAG and KIM-1 values compared to controls, but that there was no difference in urinary NGAL (22). In another study, it was demonstrated that obese children and adolescents had reduced nitric oxide (NO) levels and increased urinary isoprostanes when compared to normal weight controls (23). We found that urinary netrin-1 is significantly elevated in obese patients without microalbuminuria when compared to controls. However, there is a need for prospective studies in this regard.

Recent evidence supports the hypothesis that reduced insulin sensitivity and hyperinsulinemia are among the most important factors leading to renal injury (24). In a recent study, NAG and KIM-1 were not different in obese patients when checked for impaired glucose tolerance and insulin resistance (23). In another study, reduced NO levels, increased urinary isoprostanes, and blood pressure measurements were all found to be related to insulin resistance (23). Furthermore, we found that urinary netrin-1 level was higher in normotensive obese subjects with insulin resistance when compared to those without insulin resistance. Also, no differences were observed between these subjects for microalbuminuria. In previous studies, it was shown that netrin-1 protein is induced in proximal tubular epithelial cells and excreted in urine during diabetes before microalbuminuria onset both in animal models and in humans (12,25). In our study, the subjects did not have diabetes mellitus or hyperglycemia. Therefore, we believe that the increased netrin-1 in our patients probably was a reflection of the complications of hyperinsulinemia-induced tubular changes. In addition, there were positive correlations only between urinary netrin-1 and fasting glucose, fasting insulin, and HOMA-IR in our study. These data support the idea that hyperinsulinemia and insulin resistance seem to be effective factors for netrin-1 excretion in obese children.

Netrin-1 has a molecular mass of 72 KDa. Therefore, it is unlikely that it is filtered by glomerules under normal conditions. However, netrin-1 may be filtered after renal injury as this is known to cause changes in the filtration barrier (25). In our study, we observed no differences neither in eGFR nor in microalbuminuria between obese and control groups. This finding indicates that increased levels of urinary netrin-1 in obese patients may come from a proximal tubular source. Also, there were no differences in FE_{Na}, FE_K, and TPR values between obese and control groups. It seems that the changes in proximal tubules are detectable by increase in urinary netrin-1 even before the indicators of known proximal tubular function are affected. Thus, the findings of this study confirmed our hypothesis and showed that netrin-1 excretion in urine is increased in obese children. However, prospective studies are required to investigate whether netrin-1 reflects tubular dysfunction due to obesity.

This study has some limitations. The patient number was low. This is a cross-sectional study. Therefore, there is a need for larger prospective studies to confirm the results. We did not check other tubular injury markers like NAG, KIM-1, NGAL. Also, if there was another group with albuminuria, this would strengthen our results. We have tried to overcome the probable false results of ELISA by performing all samples in duplicates.

Obesity has a great influence on ESRD, and it can be either the cause of renal alterations and kidney injury or an aggravating factor in patients with diabetes. Netrin-1 could be a promising early biomarker of kidney injury. For showing tubular damage in obese children, urinary netrin-1 may be a new marker that can increase earlier than conventional markers of renal injury such as microalbuminuria. Insulin resistance and hyperinsulinemia seem to be related to the urinary level of netrin-1. A prospective study is needed to examine the clinical usefulness of urinary netrin-1 excretion in the early tubular injury in obese children.

Ethics

Ethics Committee Approval: Our ethics committee approved the study protocol, Informed Consent: All patients provided written informed consent.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Duygu Övünç Hacıhamdioğlu, Bülent Hacıhamdioğlu, Design: Duygu Övünç Hacıhamdioğlu, Bülent Hacıhamdioğlu, Data Collection or Processing: Duygu Övünç Hacıhamdioğlu, Bülent Hacıhamdioğlu, Demet Altun, Tuba Müftüoğlu, Analysis or Interpretation: Duygu Övünç Hacıhamdioğlu, Bülent Hacıhamdioğlu, Tuba Müftüoğlu, Ferhan Karademir and Selami Süleymanoğlu, Literature Search: Duygu Övünç Hacıhamdioğlu, Demet Altun, Writing: Duygu Övünç Hacıhamdioğlu, Bülent Hacıhamdioğlu.

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Original Article

Anti-Müllerian Hormone and Inhibin-A, but not Inhibin-B or Insulin-Like Peptide-3, may be Used as Surrogates in the Diagnosis of Polycystic Ovary Syndrome in Adolescents: Preliminary Results

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ABSTRACT

Objective: Polycystic ovary syndrome (PCOS) is a common endocrine problem in adolescents with an increasing prevalence of 30%. Pursuing new biomarkers with high specificity and sensitivity in the diagnosis of PCOS in adolescents is currently an active area of research. We aimed to investigate the diagnostic value of anti-Müllerian hormone (AMH), insulin-like peptide-3 (INSL3), inhibin-A (INH-A), and inhibin-B (INH-B) in adolescents with PCOS and also to determine the association, if any, between these hormones and clinical/laboratory findings related with hyperandrogenism.

Methods: The study group comprised 53 adolescent girls aged between 14.5 and 20 years who were admitted to our outpatient clinic with symptoms of hirsutism and/or irregular menses and diagnosed as having PCOS in accordance with the Rotterdam criteria. Twenty-six healthy peers, eumenorrheic for at least two years and body mass index-matched, constituted the controls. Fasting blood samples for hormones [luteinizing hormone (LH), follicle-stimulating hormone (FSH), dehydroepiandrosterone-sulfate (DHEAS), androstenedione (D4-A), total/free testosterone (T/fT), sex hormone binding globulin (SHBG), AMH, INSL3, INH-A, INH-B] were drawn after an overnight fast.

Results: In the PCOS group, 83% of the subjects were oligomenorrheic/amenorrheic and 87% had hirsutism. The LH, LH/FSH ratio, total T, fT, free androgen-index (FAI), DHEAS levels were significantly higher ($p=0.005$, $p=0.042$, $p=0.047$, $p<0.001$, $p=0.007$, $p=0.014$, respectively) and SHBG was significantly lower ($p=0.004$) in PCOS patients as compared to the controls. Although the INSL-3 and INH-B levels showed no difference between the groups ($p>0.05$), AMH and INH-A levels were found to be significantly higher in the PCOS group compared to the controls ($p<0.001$, $p<0.001$, respectively). In multiple linear regression analysis, WC SDS ($p=0.028$), logD4-A ($p=0.033$), logSHBG ($p=0.031$), and total ovarian volume ($p=0.045$) had significant effects on AMH levels, and LH ($p=0.003$)

WHAT IS ALREADY KNOWN ON THIS TOPIC?

One of the important biomarkers used to confirm the diagnosis of polycystic ovary syndrome (PCOS) and to manage the treatment process in adults and adolescent patients is anti-Müllerian hormone (AMH). However, new biomarkers (inhibin-A, inhibin-B, and insulin-like peptide-3) that may be used in the diagnosis and follow-up have also been found and are in the phase of investigation.

WHAT THIS STUDY ADDS?

The levels of insulin-like peptide-3 and inhibin-B were not found to have diagnostic values in adolescents with PCOS; however, it was shown that inhibin-A could be used as a new biomarker in addition to AMH.

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on INH-A levels. In receiver-operating characteristic analysis, the cut-off values for AMH and INH-A were 6.1 ng/mL (sensitivity 81.1%) and 12.8 pg/mL (sensitivity 86.8%), respectively, to diagnose PCOS. When AMH and INH-A were used in combination, the sensitivity (96.2%) increased.

Conclusion: INSL3 and INH-B were not found to have diagnostic value in adolescents with PCOS. On the other hand, it was shown that INH-A could be used as a new diagnostic biomarker in addition to AMH.

Keywords: Adolescent, anti-Müllerian hormone, inhibin-A, inhibin-B, insulin-like peptide-3, polycystic ovary syndrome

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Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine problems in women in their reproductive period (1). Although the prevalence of PCOS is 5-10% in adulthood, it may increase up to 30% in adolescence (2,3). It is thought that this reported increased prevalence in adolescents shows a variance depending on the criteria used for the diagnosis of PCOS and arises from the polycystic appearance of the ovaries which may be observed in adolescents or from menstrual irregularities which are expected in anovulatory cycles (2). It has been shown that this rate may even increase up to 50% in obese adolescents (4).

PCOS is a syndrome rather than a disease, and it has a wide variety of symptoms and clinical presentations. The classical findings include chronic anovulation, clinical or laboratory hyperandrogenism, and a typical polycystic appearance of the ovaries on ultrasonographic examination. According to the Rotterdam criteria specified in 2003, at least two of these criteria should be present to make a diagnosis of PCOS (5,6). However, it is known that the reference range of androgen levels in adolescents is different from those of adults, that the multicystic/polycystic appearance of the ovaries may be encountered in healthy girls during this period, that anovulatory cycles may be observed for a long time after menarche, and finally, that findings including acne are observed frequently in adolescent girls (7,8). Therefore, pursuing a biomarker with a high specificity and sensitivity in the diagnosis of PCOS in adolescents is currently an active area of research.

One of the important biomarkers used to confirm the diagnosis of PCOS and to manage the treatment process in adult and adolescent patients is the anti-Müllerian hormone (AMH). However, it is possible that other biomarkers, many of which are still in the phase of investigation, may be used in the diagnosis and follow-up of PCOS. One of these is insulin-like peptide-3 (INSL3) which is a member of the relaxin/insulin family. INSL3 is produced by testicular Leydig cells in men (9). In women, it is synthesized in the ovary, particularly by the theca interna cells of antral follicles as well as by the corpora lutea and ovarian stroma, and INSL3 levels seem to reflect gonadal function (10,11). INSL3 levels are similar in prepubertal and postpubertal periods, but a significant increase is observed after pubertal onset (12). Some studies show that INSL3 expression appears to change with follicle development; it is present at higher levels in small antral follicles and at lower levels as follicles become preovulatory, which suggests a correlation with follicular maturation (13,14). In contrast, Hagen et al (12) reported that INSL3 levels better reflect products from large follicles rather than products from small follicles. These authors also state that INSL3 is a specific marker for theca cells surrounding larger follicles. There are studies reporting that INSL3 is significantly increased in women with PCOS (15,16). INSL3 levels have also been investigated in healthy adolescents (12,17), but no study has investigated the diagnostic value of INSL3 in adolescent patients with PCOS to date.

Inhibin-A (INH-A) and inhibin-B (INH-B), which are the bioactive forms of inhibins in the α-subunit, are synthesized in the granulosa cells of the ovaries (18). It has been suggested that in normal women, the intercycle rise of follicle-stimulating hormone (FSH) is responsible for the increased secretion of INH-B from the small antral follicles in the early follicular phase, while the midcycle luteinizing hormone (LH) increase stimulates INH-A secretion from the pre-ovulatory follicle (19). In the light of this information, INH-B may be expected to increase in patients with PCOS who have large numbers of small antral follicles; INH-A levels increase as the follicle grows, and therefore they may be expected to be normal or decreased in PCOS patients. However, conflicting results have been reported in studies conducted on adult women. Some studies have shown that INH-A does not change in patients with PCOS (20,21), whereas others have shown that it increases (22,23,24) or decreases (25,26,27). There are also similar controversial results related with INH-B levels (20,21,27,28,29,30). No study to date has investigated these hormones only in the adolescent age group.

In this study, we aimed to investigate the diagnostic value of AMH, INSL3, INH-A, and INH-B in adolescents with PCOS and also to explore the association between these hormones and the clinical/laboratory findings related with hyperandrogenism.

Methods

We studied 53 adolescent girls aged between 14.5-20 years who were consecutively admitted to pediatric

endocrine outpatient clinic of Istanbul Faculty of Medicine between August 2014 to August 2015, with symptoms of hirsutism and/or irregular menses, and diagnosed as having PCOS in accordance with the Rotterdam Criteria (5). Adolescents with chronic diseases, thyroid hormone dysfunction, congenital adrenal hyperplasia, tumors, genetic syndromes and other endocrine disorders, and those who used medications that might potentially influence the biomedical assessments were excluded from the study. Twenty-six healthy peers who were followed up in the well-child and adolescent health care unit and were eumenorrheic for at least two years constituted the control group. Menstrual cycles were defined as oligomenorrhea if the cycle intervals were longer than 45 days and amenorrhea if the cycle intervals were longer than 3 months. In addition, we tried to match the two groups in terms of body mass index (BMI). A detailed medical assessment and medical history were obtained from all subjects. Birth weight and length of the participants, family history of PCOS, mothers' age at the time of her first menstruation, and family history for type 2 diabetes were also recorded. Written consent was obtained from all parents and participants. The study was approved by the Ethics Committee of İstanbul University Faculty of Medicine (no. 2421).

Clinical Evaluation

Weight and height were measured by the same physician (A.Y.) in all subjects using a wall-mounted calibrated Harpenden stadiometer (Holtain Ltd., Crymych, UK) and an electronic scale (sensitivity at 0.1 kg level). BMI was calculated using the following formula: BMI=weight (kg)/ height (m^2). Waist circumference (WC) was measured at the midpoint between the lower margin of the last palpable rib and the top of the iliac crest using a non-stretch tape. Hip circumference (HC) was measured around the widest portion of the buttocks with the tape parallel to the floor, and the waist to hip ratio (WHR) was evaluated. Standard deviation scores (SDS) of these measurements were calculated using national data (31,32,33). The Ferriman-Gallwey (FG) scoring method was used to define clinical hyperandrogenism (34).

Laboratory Evaluation and Biochemical Assays

Fasting blood samples for glucose, insulin, LH, FSH, dehydroepiandrosterone sulfate (DHEAS), androstenedione (D4-A), total testosterone (T), free testosterone (fT), sex hormone-binding globulin (SHBG), AMH, INSL3, INH-A, and INH-B were drawn between 08:00-08:30 a.m., after an overnight fast. Basal 17-hydroxyprogesterone (17OHP) and cortisol levels were measured to exclude an adrenal enzyme defect; free thyroxine (T_4) and thyroid-stimulating hormone (TSH) levels were measured to exclude a thyroid hormone defect, and prolactin level was measured to exclude intracranial pathologies. After separation, serum samples were frozen immediately and stored at -80 °C until they were assayed. The free androgen index (FAI) was calculated using T and SHBG values [FAI=100x(T/SHBG)].

Serum LH and FSH concentrations were measured using an immunochemiluminometric assay; DHEAS and T levels were measured using a radioimmune assay (RIA) (Diagnostic Products Corporation, Los Angeles, CA, USA). D4-A levels were determined using a solid-phase, competitive chemiluminescent enzyme immunoassay (Siemens Healthcare Diagnostics Technical Products, USA). 17OHP and serum cortisol levels were measured using RIA and DIA immunoassays (Beckmann Coulter Company, Marseille, France and S.A. Nivelles, Belgium, respectively). SHBG levels were estimated by IRMA (Roche Diagnostic, Rotkreuz, Switzerland). Free testosterone levels were measured using an RIA (Beckman Coulter Company, Prague, Czech Republic). Serum INH-A, INH-B, AMH (AL105-I), and INSL3 (SED873Hu) levels were determined by an enzyme-linked immunosorbent assay (ELISA) (Beckman Coulter Company, Webstar, USA, and USCN-life, Houston, USA). LH and FSH had an intra-assay coefficient of variation (CV) of 4.8-7.5% and interassay CV of 5.4-10.7%, respectively. DHEAS had an intra-assay CV of 4.5 and an interassay CV of 5.5%. Testosterone had an intra-assay CV of 4.5% and an interassay CV of 6.4%. For the D4-A assay, the intra-assay CV was 3.2-9.4% and interassay CV was 4.1-15.6%. For the cortisol assay, the intra-assay CV was 5.2% and interassay CV was 8.7%. The 17-OHP assay had intra-assay and interassay CVs of 5%. For the SHBG assay, the intra-assay CV was 2% and interassay CV was 8.3%. The INH-A assay had an intra-assay CV of 3.4-5.6% and an interassay CV of 5.5-6.7%, and the INH-B assay had an intra-assay CV of 2.9-4.5% and an interassay CV of 5.5-7.5%. Values for the AMH assay were 1.9-4.0% (intra-assay CV) and 4.5-6.0% (interassay CV). The INSL3 assay had intra-assay and inter-assay CVs of <10% and <12%, respectively.

Pelvic Ultrasound

Ultrasound (US) examinations were performed on the same day as the hormonal and biochemical determinations. Transabdominal pelvic US scans were performed in the PCOS group prospectively by the same experienced pediatric radiologist (O.B.E.) who was blinded to the clinical and laboratory findings of the subjects. No US was done in the control group. US was performed using a conventional full bladder Logiq 6 US scanner (General Electric Co., Milwaukee, WI, USA) and a 5-MHz convex-array broad-band transducer or a 7.5-MHz linear-array small parts transducer,

depending on age and size. The three dimensions of the uterus [total uterine length (UL), anteroposterior (AP), and transverse diameters of the corpus], endometrial thickness (ET), and the three dimensions of each ovary (longitudinal, transverse, and AP diameters) were measured. Uterine and ovarian volumes were calculated according to the formula for ellipsoid bodies: longitudinal diameter x AP diameter x transverse diameter x 0.52. An ovary was defined as polycystic if there were 12 follicles or more, each 2-9 mm in diameter (35).

Statistical Analysis

SPSS version 15 (Chicago, IL, USA) was used for statistical analyses. Results are given as mean ± SD or median [minimum-maximum]. Normality was assessed using the Kolmogorov-Smirnov test. The normality of continuous variables was examined using three different methods including variability coefficient, Kolmogorov-Smirnov, and skewness-kurtosis values; if two of these three methods showed normal distribution, the distribution was considered compatible with normal distribution.

Parametric and nonparametric tests were used for inter-group comparisons. Skewed data (AMH, INH-A, INH-B, T, fT, SHBG, 17OHP, D4-A) were transformed to normal distributions by calculating normal logarithms and natural logarithms. Chi-square test was used for categorical variables, while student's t-test and Pearson's correlation analysis was applied for continuous variables in independent groups. The Mann-Whitney U test was used for continuous variables that did not show normal distribution. The linear regression model was applied using backward stepwise method in the patients with PCOS considering AMH, and INH-A as a dependent variable.

The candidate biomarkers that showed a significant difference in inter-group comparisons were examined using the receiver operating characteristics (ROC) curve analysis and their diagnostic values (sensitivity and specificity) were calculated. When the type-1 error level was below 5% in the evaluation of area under the curve (AUC), the diagnostic value of the test was considered statistically significant. The net sensitivity and specificity rates of the combined use of AMH and INH-A in the diagnosis of PCOS were calculated (36).

Results

Thirty-four percent of the patients with PCOS (n=18) were amenorrheic, 49% (n=26) were oligomenorrheic, and 17% (n=9) were eumenorrheic. Hirsutism was present in 87% of the patients with PCOS (n=46), acne was present in 43% (n=23), alopecia in 25% (n=13), and acanthosis nigricans in 49% (n=26). The distribution of subjects with obesity/overweight and normal body weight was similar in the PCOS group (57%) and control group (50%) (BMI-matched) (p>0.05).

Although the mean age of the PCOS group was slightly higher compared with the control group (p=0.001), the age at the time of menarche was similar in both groups (p=0.397). The birth weight SDS, birth length SDS, and BMI SDS values were similar in both groups (p>0.05). The WC SDS value was statistically significantly higher in the PCOS group (p<0.001). The anthropometric measurements of the patient and control groups are shown in Table 1.

The hormone levels of the PCOS and control groups are presented in Table 2. The LH, LH/FSH ratio, total T, fT, FAI, DHEAS levels were significantly higher in the PCOS group (p=0.005, p=0.042, p=0.047, p<0.001, p=0.007, p=0.014,

Table 1. Clinical and anthropometric findings of the polycystic ovary syndrome patients and controls

	PCOS patients n=53	Controls n=26	p
Age (years)	16.72±1.41	15.18±2.00	0.001
Menarcheal age (years)	12.53±1.36	12.27±1.09	0.397
Birth weight SDS	-0.28 [-3.66–3.79]	0.08 [-2.58–1.76]	0.949
Birth length SDS	0.55 [-2.90–1.66]	0.45 [-4.27–2.78]	0.466
Weight SDS	1.52 [-2.88–6.68]	0.33 [-2.46–4.02]	0.054
Height SDS	-0.10 [-3.30–2.89]	-0.49 [-2.57–1.50]	0.278
BMI SDS	1.40 [-1.74–9.20]	0.78 [-1.99–3.14]	0.096
WC SDS	3.59 [0.06–10.47]	0.85 [-0.77–4.00]	<0.001
FG score	13.50 [6.00–24.00]	-	-

The results are expressed as mean ± standard deviation or median [minimum-maximum] for log transformed data, unless stated otherwise. p<0.05 was significant.
PCOS: polycystic ovary syndrome, SDS: standard deviation score, BMI: body mass index, WC: waist circumference, FG: Ferriman-Gallwey

respectively); SHBG was found to be significantly lower in the PCOS group (p=0.004). Although the INSL-3 and INH-B levels showed no difference between the groups (p>0.05), the AMH and INH-A levels were found to be significantly higher in the PCOS group compared with the control group (p<0.001, p<0.001, respectively).

Correlation Analysis

Anthropometric measurements: INSL3 level showed no significant correlation with the anthropometric measurements, whereas the AMH level had a positive correlation with WC SDS and WHR (r=0.305, p=0.008; r=0.240, p=0.038), the INH-B level demonstrated negative correlations with BMI SDS, WC SDS, WHR (r=-0.426, p=0.001; r=-0.377, p=0.001; r=-0.242, p=0.034, respectively), and the INH-A level had a positive correlation with WC SDS (r=0.285, p=0.013). FAI was found to positively correlate with the FG score, BMI SDS, WC SDS, and WHR (r=0.623, p<0.001; r=0.535, p<0.001; r=0.433, p<0.001; r=0.299, p=0.014, respectively).

Hormones: There was a negative correlation between the INSL3 level and INH-A (r=-0.296, p=0.009). AMH was found to significantly correlate with LH, DHEAS, fT, D4-A, and INH-A (r=0.255, p=0.032; r=0.288, p=0.014; r=0.572, p<0.001; r=0.415, p=0.004; r=0.385, p=0.001, respectively). The INH-A level significantly correlated with LH, LH/FSH ratio, SHBG, DHEAS, and cortisol levels in addition to AMH and INSL3 (r=0.313, p=0.008; r=0.350, p=0.003; r=-0.261, p=0.031; r=0.347, p=0.003; r=0.359, p=0.002, r=0.385, p=0.001; r=-0.296, p=0.009, respectively). The INH-B level was found to significantly correlate only with FSH (r=0.247, p=0.035).

Ultrasonographic findings: AMH was found to significantly correlate with left ovarian volume and total ovarian volume (r=0.438, p=0.002; r=0.346, p=0.019, respectively). INH-A significantly correlated with right ovarian volume and total ovarian volume (r=0.333, p=0.024; r=0.315, p=0.033, respectively).

Regression Analysis

In the PCOS group, multiple linear regression analysis was performed to explore the effect of WC SDS, FSH, logD4-A, logSHBG, logT, and total ovarian volume on the level of AMH. Factors that had an effect on the level of AMH included (adjusted R2=0.284) WC SDS (β=-0.058, p=0.028), logD4-A (β=0.664, p=0.033), logSHBG (β=0.012, p=0.031), and total ovarian volume (β=-0.495, p=0.045)

Table 2. Laboratory findings in polycystic ovary syndrome patients and in the control group

	PCOS patients n=53	Controls n=26	p
LH (mIU/mL)	10.60 [3.00–79.10]	7.18 [1.57–33.93]	0.005
FSH (mIU/mL)	5.27±1.76	5.01±2.10	0.597
LH/FSH	2.30 [0.63–11.30]	1.38 [0.51–4.74]	0.042
T (ng/mL)	0.44 [0.16–2.21]	0.36 [0.05–1.91]	0.047
fT (pg/mL)	2.68 [0.83–6.32]	0.95 [0.49–2.30]	<0.001
FAI	6.75 [0.97–23.65]	3.00 [0.22–36.65]	0.007
DHEA-S (µg/dL)	301.31±122.11	222.37±127.62	0.014
SHBG (ng/mL)	7.46 [2.37–37.67]	14.28 [4.67–26.78]	0.004
17OHP (ng/mL)	1.19 [0.18–3.97]	-	-
D4-A (ng/mL)	4.50 [1.41–10.00]	-	-
AMH (ng/mL)	11.02 [1.66–50.60]	4.06 [0.93–11.96]	<0.001
INH-A (pg/mL)	29.19 [1.68–307.10]	2.00 [1.34–146.10]	<0.001
INH-B (pg/mL)	61.49 [3.44–273.50]	91.04 [16.05–153.60]	0.236
INSL3 (pg/mL)	1260.76±285.37	1359.26±236.10	0.132
Total ovarian volume (mL)	27.15 [6.80–46.00]	-	-
L ovarian volume	13.00 [2.80–28.00]	-	-
R ovarian volume	13.60 [3.90–29.00]	-	-

The results are expressed as mean ± standard deviation or median [minimum-maximum] for log transformed data, unless stated otherwise. PCOS: Polycystic ovary syndrome, LH: luteinizing hormone, FSH: follicle-stimulating hormone, T: total testosterone, fT: free testosterone, FAI: free androgen index, DHEAS: dehydroepiandrosterone sulfate, SHBG: sex-hormone binding globulin, 17OHP: 17-hydroxyprogesterone, D4-A: androstenedione, AMH: anti-Müllerian hormone, INH-A: inhibin-A, INH-B: inhibin-B, INSL3: insulin-like peptide-3

(Table 3). Multiple linear regression analysis was also performed to explore the effect of logAMH, INSL3, LH, logSHBG, and DHEAS on the level of INH-A. The only factor that had an effect on the level of INH-A (adjusted R2=0.157) was LH (β=2.023, p=0.003) (Table 4).

Receiver Operating Characteristics Analysis for Anti-Müllerian Hormone and Inbibin-A

ROC curve analyses were performed to determine the ability of AMH and INH-A to distinguish between adolescents with PCOS and controls (Figure 1): for AMH-AUC of 0.88, p<0.001, 95% CI: [0.80-0.96]; for inhibin-A -AUC of 0.74, p=0.001, 95% CI: [0.61-0.87], respectively. The cut-off value for AMH was 6.1 ng/mL, and the cut-off value for INH-A was 12.8 pg/mL to make a diagnosis of PCOS. With these cut-off values, AMH had a specificity of 92.3% and a sensitivity of 81.1% in the diagnosis of PCOS. When INH-A was used, the specificity and sensitivity were 69.2% and 86.8%, respectively. When AMH and INH-A were used in combination, the specificity and sensitivity were found as 65.4% and 96.2%, respectively (Figure 2).

Discussion

Our study is the first study in which INH-A, INH-B, and INSL-3 levels, together with AMH levels, were examined in adolescents with PCOS. In addition, we believe it is also important to note that in this study, the cut-off values with high sensitivity and specificity rates for AMH and INH-A have been determined.

WC measurement and WHR were reported to be more contributory compared with BMI in the diagnosis of metabolic syndrome (37). Visceral adiposity is a frequent finding in patients with PCOS, and this leads to hyperandrogenemia by way of insulin resistance (38,39). In our study, there was no difference between the PCOS and control groups in terms of obesity-overweight and normal weight distribution. WC SDS and WHR levels, which indicate increased visceral adiposity in adolescents with a diagnosis of PCOS, were found to be higher in the PCOS group compared with controls. Cortet-Rudelli et al (40) reported a positive correlation between FAI and the rate of visceral adiposity. A positive correlation was also shown between FAI and WC SDS in our study; AMH and INH-A were also found to correlate with WC SDS.

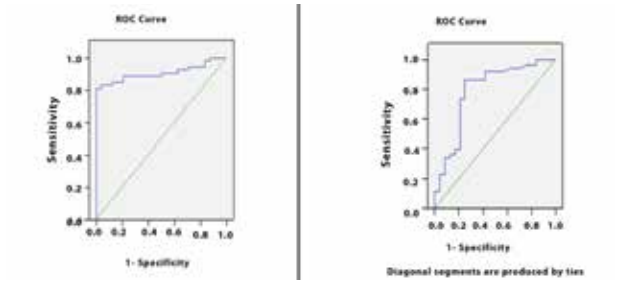

Figure 1. Receiver operating characteristics (ROC) curves of anti-Müllerian hormone (left) and inhibin-A (right). The sensitivity (y axis) is plotted against the specificity (x axis)

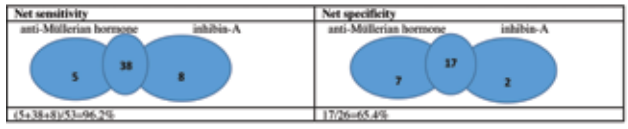

Figure 2. Net sensitivity and specificity when anti-Müllerian hormone and inhibin-A were used together

Table 3. Multiple linear regression analysis of the factors associated with logarithmic anti-Müllerian hormone in polycystic ovary syndrome group (adjusted R2=0.284)

	B	SE	t	p	OR (95% confidence interval)
WC SDS	-0.058	0.025	-2.300	0.028	-0.110-0.007
logD4-A	0.664	0.299	2.219	0.033	0.055–1.273
logSHBG	0.012	0.005	2.249	0.031	0.001–0.023
Total ovarian volume	-0.495	0.237	-2.089	0.045	-0.977–-0.013

The variables entered in the first step of various models included waist circumference standard deviation score (WC SDS), logarithmic androstenedion (logD4-A), logarithmic sex-hormone binding globulin (logSHBG), and total ovarian volume. Confidence interval 95%, p<0.05 was significant. SE: standard eror, OR: odds ratio

Table 4. Multiple linear regression analysis of the factors associated with logarithmic inhibin-A in polycystic ovary syndrome group (adjusted R^2=0.157)

	B	SE	t	p	OR (95% confidence interval)
LH	2.023	0.648	3.122	0.003	0.719-3.328

The variables entered in the first step of various models included logarithmic anti-Müllerian hormone (logAMH), insulin-like peptide-3 (INSL3), luteinizing hormone (LH), logarithmic sex-hormone binding globulin (logSHBG), and dehydroepiandrosterone sulfate (DHEAS). Confidence interval 95%, p<0.05 was significant. SE: standard eror, OR: odds ratio

In the literature, results related to the diagnostic value of AMH in adolescents are conflicting. In most studies, AMH levels have been found to be increased in patients with PCOS (41,42,43). However, some publications have shown that the increase in AMH levels was not significant (44). In our study, AMH level was found to be significantly higher in patients with PCOS than in controls. In a meta-analysis study, various values were specified for the cut-off value of AMH. Sensitivity increased, but specificity decreased below 50% if this value was <3 ng/mL. When the cut-off value was accepted as >5 ng/mL, the specificity increased above 80% and the sensitivity decreased below 70% (6). In these studies which were conducted on adult women, the cut-off value was specified to be about 4.7 ng/mL. Few studies have proposed a cut-off value for AMH in adolescents. Deveer et al (45) had estimated the cut off value of AMH for adolescents with PCOS as 6.6 ng/mL. In this present study, a cut-off value of 6.1 ng/mL was shown to have a high sensitivity and specificity.

Different results have been reported in studies that examined androgens as they related with AMH in patients with PCOS. Some studies have shown that AMH closely correlated especially with testosterone (43,46), and others have shown that it correlated with D4-A and FAI (47). In our study, AMH was found to significantly correlate with androgens such as DHEAS, fT, D4-A.

INSL3 has been suggested as a laboratory test that can be used in the diagnosis of PCOS (16,48). However, these studies were conducted with adult women. In our study, adolescent girls with PCOS were compared with healthy adolescent girls, and INSL3 was not significantly different in the two groups. To date, only two studies have investigated the levels of INSL3, but these studies were conducted on healthy adolescents. Pelusi et al (17) showed that INSL3 was higher in healthy adolescents with anovulatory cycles compared with adolescents who had ovulatory cycles, but the number of subjects with anovulatory cycles was considerably limited in their study. In a study which included peripubertal healthy girls, it was proposed that INSL3 was released especially from large antral follicles (12). Accordingly, it may be thought that INSL3 has no place in the diagnosis of PCOS, but it should be noted that this study was also conducted with a limited number of subjects. In some studies, it has been reported that INSL3 was related with androgens that originate from the ovary. However, these studies were conducted on late adolescents and adults (12,15). In our study, it was also found that INSL3 positively correlated with the level of T, albeit insignificantly.

Inhibins are hormones released in the ovaries during follicular development, and their effects in patients with PCOS are still a subject of investigation. No studies have been conducted with adolescents with PCOS in this area. Studies conducted with adults have yielded controversial results. While some have shown that INH-A does not change in patients with PCOS (20,21), other studies have shown an increase (22,23,24), while some other studies reported a decrease (25,26,27). Some studies have shown that INH-B level increases in patients with PCOS (28), whereas others have shown that it decreases (30). Overall, results generally indicate that the INH-B level does not change markedly in PCOS patients (20,21,23,27,29). It has been shown that the levels of INH-A are low in the follicular phase of ovulation, increase just after ovulation, and increase further as the follicle develops (49,50). INH-B is released from small antral cells during the follicular phase (19,21). It is to be expected that the level of INH-A, which is related to increased LH level, and the level of INH-B, which is related to increased FSH level, are variable in patients with PCOS since LH and FSH levels are also variable in these patients who mostly have an increased LH/FSH ratio with LH predominance. These studies have been conducted on women with PCOS according to menstrual cycle phases, considering the periods of the menstrual cycle (21). However, patients with PCOS generally have LH predominance. Therefore, it may be expected that the level of INH-B, which is induced by FSH, is not increased and INH-A is increased together with increased LH secretion independent of the cycle in these patients who are generally oligo-/amenorrheic. Similarly, Pigny et al (23) found the level of pro-αC INH-A, which has both mature and immature forms in patients with PCOS, to be significantly increased. In the adolescents with PCOS in our study, the level of INH-A was found to be considerably increased and its sensitivity was high. In addition, the level of INH-B did not show a marked difference, a finding in accordance with many other studies (20,21,23,27,29). This finding may also be related to the fact that our patient group was especially composed of oligo-/amenorrheic adolescents. We think that studies comparing the INH-A and INH-B levels between eumenorrheic and oligo-/amenorrheic patients with PCOS may explain the conflicting results related with these two hormones.

The inverse correlation of the INH-B level with BMI has been shown in many studies, but it has also been pointed out that this correlation is not a specific finding for patients with PCOS (23,40). In our study, the patients and control groups were specifically matched in terms of BMI, and it was found that INH-B strongly inversely correlated with BMI SDS and WC SDS, while INH-A positively correlated with WC SDS. In a study conducted by Pigny et al (23), INH-A level inversely correlated with BMI, but its correlation with visceral adiposity was not mentioned. More studies are needed in this area.

In our study, INH-B was found to correlate with FSH level. This correlation may be considered an expected finding for INH-B which shows an increase with FSH secretion. However, some studies have found an inverse correlation between these two hormones (20). The correlation of INH-A with LH level and LH/FSH ratio confirmed the findings of another study (20) and regression analysis also showed the effect of LH on INH-A in our study. Correlation analysis demonstrated that INH-A also correlated with SHBG and DHEAS. Some studies have claimed that there is no correlation between INH-A and androgens in patients with PCOS, but in those studies, the comparisons were made by examining only some androgens (20,21,26). Larger scale studies are also needed in this area.

One of the limitations of our study was the fact that the ages were different in the patient and control groups. However, the fact that BMI SDS values and age of menarche were similar in the two groups, partially eliminates this limitation. Also, our control group consisted of adolescents who were eumenorrheic for at least two years. BMI-matched groups were selected to enable accurate evaluation of the hormones, because it is known that INH-B levels correlate with BMI (21,30).

In conclusion, the results of this present study indicate that the levels of INSL3 and INH-B do not have diagnostic value in adolescents with PCOS. On the other hand, it was shown that INH-A could be used as a new diagnostic biomarker in addition to AMH. Currently, we need more large-scale studies to identify biomarkers that could be helpful in adolescent cases where the diagnosis of PCOS is not definite.

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Ethics

Ethics Committee Approval: The Ethics Committee of İstanbul University Faculty of Medicine (no. 2421), Informed Consent: Written consent was obtained from all parents and participants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Aylin Yetim, Firdevs Baş, Feyza Darendeliler, Design: Aylin Yetim, Firdevs Baş, Feyza Darendeliler, Data Collection or Processing: Aylin Yetim, Firdevs Baş, Feyza Darendeliler, Oğuz Bülent Erol, Analysis or Interpretation: Aylin Yetim, Firdevs Baş, Feyza Darendeliler, Gülnaz Çığ, Literature Research: Aylin Yetim, Firdevs Baş, Feyza Darendeliler, Çağcıl Yetim, Ahmet Uçar, Writing: Aylin Yetim, Firdevs Baş, Feyza Darendeliler, Çağcıl Yetim, Ahmet Uçar.

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Original Article

Hidden Toxicity in Neonatal Intensive Care Units: Phthalate Exposure in Very Low Birth Weight Infants

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ABSTRACT

Objective: To determine exposure to endocrine-disrupting phthalates in preterm infants in neonatal intensive care units (NICU).

Methods: Urine samples (n=151) from 36 preterm infants (<32 weeks of gestation and/or <1500 g of birth weight) were collected on the first 3 days of admission to the NICU and biweekly thereafter. Diethylhexyl phthalate contents of indwelling medical devices used in various procedures and the concentrations of phthalate metabolites in the urine samples were analyzed. The relationships between urinary excretion, exposure intensity, postnatal age and birth weight were examined.

Results: The mean gestational age and mean birth weight of the study infants were 28.9±1.5 weeks and 1024±262 g, respectively. Diethylhexyl phthalate was detected in umbilical catheters, endotracheal tubes, nasogastric tubes, and nasal cannula. Monoethylhydroxyhexyl phthalate (MEHHP) was the most frequently detected metabolite (81.4%); its concentration increased during the first 4 weeks and then started to decrease but never disappeared. Patients who did not need indwelling catheters (except nasogastric tubes) after 2 weeks were classified as group 1 and those who continued to have indwelling catheters as group 2. Although not of statistical significance, MEHHP levels decreased in group 1 but continued to stay high in group 2 (in the 4th week, group 1: 65.9 ng/mL and group 2: 255.3 ng/mL). Levels of MEHHP in the first urinary samples were significantly higher in infants with a birth weight <1000 g (<1000 g: 63.2±93.8 ng/mL, ≥1000 g: 10.9±22.9 ng/mL, p=0.001).

Conclusion: Phthalate metabolites were detected even in the first urine samples of very low birth weight newborns. Phthalate levels were higher in the first weeks of intensive invasive procedures and in preterm infants with a birth weight less than 1000 g. MEHHP was the most frequently detected metabolite and could be a suitable biomarker for the detection of phthalate exposure in preterm infants.

Keywords: Newborn, preterm, phthalate, exposure, neonatal intensive care units

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Phthalates are endocrine disruptors and normally should not be present in humans.

WHAT THIS STUDY ADDS?

High amounts of phthalate metabolites were detected in the urine samples of neonatal intensive care unit patients and exposure was associated with the intensity and duration of invasive medical procedures.

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Introduction

Phthalates, which are used as plasticizers, increase the flexibility and durability of polyvinyl chloride (PVC) products. Diethylhexyl phthalate (DEHP) is the most commonly used plasticizer for PVC. Soft PVC can consist of a high percentage of DEHP (up to 40%) (1). Annual phthalate consumption is more than 1 million tons in Western Europe and over 3 million tons globally (2,3). Because of its widespread use, exposure of humans is virtually unavoidable. DEHP is a major component of household products, clothing, packaging, and medical devices. As DEHP is not chemically bound to plastic materials, it can be leached to the environment or directly into the body fluids, thus exposing humans via ingestion, inhalation, dermal absorption, or intravenous route.

Preterm neonates are frequently exposed to numerous medical devices in neonatal intensive care units (NICU). DEHP is a major plasticizer for medical products such as blood and total parenteral nutrition bags, feeding tubes, umbilical catheters, peripherally inserted central catheters, oxygen masks, and endotracheal tubes. The rate of DEHP release depends on the DEHP content of the plastic material, lipophilic nature and flow rate of the solution in contact with the PVC tubing, temperature during use, and storage period (4). In 2005, National Toxicology Program's Center for the Evaluation of the Risks to Human Reproduction (CERHR) suggested that DEHP exposure of infants in intensive care units may be 2-3 orders of magnitude higher than that of the general adult population and that the level of DEHP exposure may approach the lowest observed adverse effect levels in animal studies (14-23 mg/kg/day) (5).

DEHP is rapidly hydrolyzed to its monoester, monoethylhexyl phthalate (MEHP), which is a minor urinary metabolite of DEHP. MEHP is oxidized to other metabolites such as monoethylhydroxyhexyl phthalate (MEHHP) and monoethyloxohexyl phthalate (MEOHP) which are excreted in the urine in quantities several times greater than MEHP. Thus, these metabolites may be more sensitive biomarkers of DEHP exposure than MEHP. After metabolization, phthalates are rapidly excreted in the urine (90%) and feces (10%), may cross the placenta, and can be detected in breast milk and amniotic fluid (6,7,8).

Phthalates have endocrine-disrupting properties. High-level exposure to phthalates may cause fetal death, malformations, cancer, liver and kidney injury, and reproductive abnormalities in animals (9,10,11). Acute toxicity of phthalates in humans is probably low, but chronic exposure may have reproductive and developmental toxic effects. Fetal and neonatal periods are very sensitive periods for the effects of endocrine disrupters. Exposure to environmental toxic chemicals may increase the incidence of reproductive deficits. Intensive therapeutic interventions and impaired ability to excrete phthalates may explain the susceptibility of preterm infants to high DEHP exposure.

There is limited data on the exposure to DEHP for preterm infants in the NICU. Most of the published data are from animal studies and adult exposure (9,10,11,12,13). However, because of their high exposure rates to DEHP in the NICU and their limited excretion capacity, preterm infants are the most at-risk population (14,15,16,17,18). In this prospective study, we studied the levels of DEHP metabolites in preterm infants who underwent intensive therapeutic medical interventions. This is the first study showing the sequential changes in the levels of DEHP metabolites in preterm infants during their stay in the NICU.

Methods

Preterm infants with a gestational age of less than 32 weeks and/or a birth weight of less than 1500 g who stayed in the NICU of the Neonatology Section of the Department of Pediatrics of the İstanbul Faculty of Medicine for at least 2 weeks were enrolled in the study. Infants with major congenital anomalies were excluded. Urine samples were collected from all preterm infants at admission or in their first 3 days in the NICU and every 2 weeks until discharge. A total of 151 urine samples from 36 preterm infants were collected. To determine the effect of exposure intensity and length of exposure on the excretion of phthalate metabolites, two groups of patients were defined: the first group had only a feeding tube as an intervention after two weeks of life, whereas the second group had ongoing interventions using various medical devices (umbilical catheter, nasal cannula, endotracheal tube).

The institutional review board of İstanbul University İstanbul Medical Faculty approved the study protocol. Informed consent was obtained from the parents.

A glass tube was used to collect the urine samples. The tubes were placed around the orifice of the urethral meatus, supported by the diaper, and at least 1 mL of urine was recovered. Urine samples were labelled and frozen immediately at -20 °C until analyzed. After all the urine samples were collected, they were sent on dry ice to TÜBİTAK (The Scientific and Technological Research Council of Turkey) for analysis.

Urine samples were analyzed for DEHP and its 3 metabolites, MEHP, MEHHP, and MEOHP. Standard DEHP, MEHP, MEOHP, MEHHP, and MEOHP-^{13}C4 solutions were procured from LGC and Cambridge Isotopes Laboratories INC. Acetonitrile and other chemicals (HPLC grade) were purchased from Merck (Darmstadt, Germany). Stock solutions of standards (MEHP, DEHP, MEOHP, and MEHHP) and internal standard (MEOHP-^{13}C4) were prepared in acetonitrile [adapted from Kato et al (19) 2004]. Urinary phthalate metabolites were determined by liquid chromatography Tandem Gold quadruple mass spectrometry electrospray ionization (ESI)-liquid chromatography-mass spectrometry (LC-MS)/MS. Cerex system 48, pressure processor vacuum manifold equipment were used for extraction of phthalate analytes prior to LC-MS/MS analysis.

The analytical methods for measuring phthalate metabolites in urine were adapted from Blount et al (12) 2000, Kato et al (20) 2003 and Silva et al (21) 2003. For the free metabolite analysis (non glucuronidated), analytes (1 mL) were spiked with MEOHP-^{13}C4 and the phthalate metabolites were extracted from the matrix by solid phase extraction (Oasis HLB, Waters, Milford, MA). The analytes were chromatographically separated by liquid chromatography on a phenyl column (Betasil 5 µm, 50 mmx3 mm) water (acetic acid 0.1%): acetonitrile (acetic acid 0.1%) gradient and analysed by tandem mass spectrometry using electrospray ionization.

All analytes were calibrated linearly with eight standard analyte solutions and six repetitives between 1 ng/mL to 100 ng/mL. The limits of detection were calculated $3S_0$, where S_0 is the standard deviation value as the concentration approaches zero. S_0 was determined from the replicate analysis of low-level standards. The relative standard deviations were found below 15%.

All of the samples, blanks, and standards were processed identically using equipment software. Each ion of interest in the chromatogram was automatically selected and integrated. The urinary concentrations were reported in nanograms per milliliter of urine.

DEHP concentrations of unused plastic devices were also determined by gas chromatography and mass spectroscopy (Clarus gas chromatography, Perkin Elmer, Shelton, USA and Clarus 600 T mass spectroscopy, Perkin Elmer, Shelton, USA) and were reported in mg per 0.5 g of that plastic material.

Statistical Analysis

Statistical Package for Social Sciences (SPSS) 15.0 was used for statistical analysis. For descriptive methods (minimum, maximum, and median) of the two groups, Mann-Whitney U test was used. Friedman test and Wilcoxon rank test were performed for correlations of medians in more than two time periods. Correlation coefficients were calculated by Pearson's test. The p-value and confidence interval were accepted as <0.05 and 95%, respectively.

Results

A total of 36 preterm infants (15 boys, 21 girls) with a mean gestational age of 28.9±1.5 weeks (25-31 weeks) and a mean birth weight of 1024±262 g (585-1560 g) were enrolled. The gestational age of 11 patients (31%) was less than 28 weeks, 18 patients (50%) had a birth weight of less than 1000 g, and 9 patients (25%) were small for gestational age. Prolonged rupture of membranes (n=10, 28%) and preeclampsia (n=7, 19%) were the most common pregnancy complications. The majority of the patients (n=31, 86%) were born by cesarean section. Twenty seven patients (75%) had respiratory distress syndrome.

Urinary levels of MEOHP and MEHHP, which are the oxidized metabolites of DEHP, were higher than those of DEHP and MEHP and were also the most commonly detected urinary metabolites (Table 1). As all the metabolites were statistically significantly correlated with each other, the most commonly detected metabolite MEHHP was used as the biomarker of phthalate exposure in our study.

Endotracheal tubes, feeding tubes, nasal cannulas, umbilical catheters, peripherally inserted central catheters, and peripheral lines were the most commonly and long-term used plastic medical devices in our NICU. Phthalates were detected in unused nasal cannulas, feeding tubes, endotracheal tubes, and umbilical catheters. Nasal cannulas had the highest phthalate content (201.7 mg/0.5 g). Phthalate contents of feeding tubes, endotracheal tubes, and umbilical catheters were 71 mg/0.5 g, 29.1 mg/0.5 g, and 3.8 mg/0.5 g, respectively. Peripherally inserted central catheters and peripheral lines had no detectable phthalate content.

The median levels of DEHP, MEHP, MEOHP, and MEHHP are shown in Figure 1. MEHHP levels were higher in the first 2 to 4 weeks of intensive medical intervention and then started to decrease when this period of intensive exposure to plastic materials also decreased, although a small surge has been seen in the eighth week. Only 22 urine samples (14.4% of total urine samples) were collected after the eighth week of

Table 1. Detection rates and levels of phthalate metabolites (ng/mL) in urine samples

	Percent detection n (%)	Mean	SD	Median	Minimum	Maximum
DEHP	64 (42.4)	5.8	15.5	0.0	0.0	163.7
MEHP	3 (2)	13.0	112.5	0.0	0.0	1234.3
MEOHP	114 (75.5)	239.6	670.3	48.4	0.0	5841.5
MEHHP	123 (81.4)	319.5	711.2	84.0	0.0	5068.6

DEHP: diethylhexyl phthalate, MEHP: monoethylhexyl phthalate, MEOHP: monoethyloxohexyl phthalate, MEHHP: monoethylhydroxyhexyl phthalate, SD: standard deviation

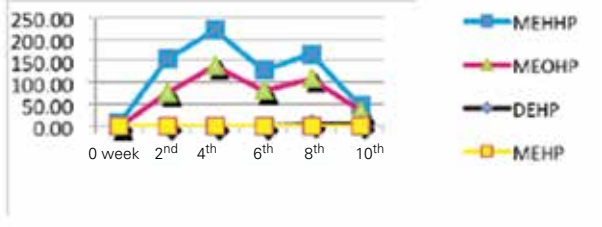

Figure 1. Median levels of phthalate metabolites (ng/mL). DEHP: Diethylhexyl phthalate, MEHP: monoethylhexyl phthalate, MEOHP: monoethyloxohexyl phthalate, MEHHP: monoethylhydroxyhexyl phthalate

the study and MEHHP levels in some of these samples were extremely higher than those in the general study population, probably accounting for the small surge seen in the eighth week. Hence, we used the first six-week levels of MEHHP in the correlations.

The median urinary levels of MEHHP according to exposure intensity and length of exposure are shown in Table 2 and Figure 2. Although there was no statistically significant difference between the groups, urinary MEHHP levels started to decrease in the first group (no further intervention) but stayed high in the second group (ongoing intervention) with high exposure intensity, extending through the sixth week of life.

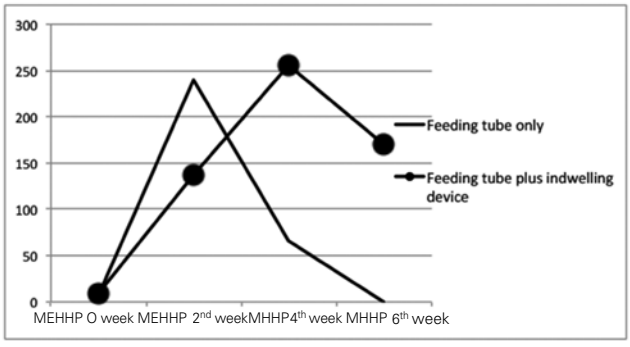

Figure 2. Comparison of median levels of monoethylhydroxyhexyl phthalate (ng/mL) according to exposure intensity and length of exposure. MEHHP: monoethylhydroxyhexyl phthalate

Table 2. Comparison of median levels of monoethylhydroxyhexyl phthalate (ng/mL) according to exposure intensity and length of exposure

Weeks	No further intervention (Feeding tube only) (n=13)	Ongoing intervention (n=23)	p
MEHHP 0 week	6.9	9.2	0.626
MEHHP 2nd week	240.5	136.5	0.745
MEHHP 4th week	65.9	255.3	0.308
MEHHP 6th week	0	169.9	-

MEHHP: monoethylhydroxyhexyl phthalate

When the urinary excretion of MEHHP levels were compared according to birth weight category, the levels in the first urinary samples of extremely low birth weight infants were significantly higher than those of infants with a birth weight of ≥1000 g (p=0.001) although the intensity of interventions were similar between the groups (Table 3).

Discussion

Phthalate exposure is widespread and unavoidable in NICU. Various medical devices, like feeding tubes, endotracheal tubes and umbilical catheters, contain phthalates as plasticizers. DEHP is the most commonly used phthalate derivative. While the major source of human exposure is ingestion of contaminated food or exposure via the dermal route, in NICU, the newborn infants are exposed to very high doses of phthalates during medical procedures such as mechanical ventilation, parenteral nutrition, or blood transfusion (14). In adults, approximately 80-90% of urinary metabolites of phthalates are conjugated to glucuronic acid and easily excreted, but in newborns and preterm infants, this conjugation pathway is immature (15). Furthermore, renal clearance is reduced due to a low glomerular filtration rate, and this may also increase the toxicity risk of phthalates. In the present study, we prospectively and sequentially studied the excretion of phthalate metabolites in the urine samples of preterm infants during their stay in our level III NICU.

Most of the previous studies have been performed on animals and adults. There are only a few studies in newborn infants and most of them are cross-sectional with a limited number of patients (16,17,18). The first study was reported by Calafat et al (16) who collected 41 urine samples from 6 preterm patients and showed increased DEHP metabolites (MEHP, MEOHP, and MEHHP) in urine samples confirming that newborns who underwent intensive invasive procedures in NICU were exposed to higher concentrations of DEHP than the general population. Green et al (17) showed in 54 newborns in NICUs that intensive use of DEHP-containing medical devices resulted in higher exposure to DEHP as reflected by increased MEHP in their urine samples. In a follow-up

Table 3. Comparison of exposure intensity and urinary monoethylhydroxyhexyl phthalate levels (ng/mL) of patients on admission to the neonatal intensive care units (the first 3 days) according to birth weight category

	<1000 g			≥1000 g			p
Endotracheal tube, n (%)	15 (83.3)			12 (66.6)			0.443
Umbilical catheter, n (%)	18 (100)			17 (94.4)			0.999
Nasal cannula, n (%)	6 (33.3)			8 (44.4)			0.773
Nasogastric tube, n (%)	18 (100)			18 (100)			-
Urinary MEHHP levels	Mean	SD	Median	Mean	SD	Median	
	63.2	93.8	22.1	10.9	22.9	0.0	0.001

MEHHP: monoethylhydroxyhexyl phthalate, SD: standard deviation

report, the same group of researchers investigated the urinary excretion of two additional metabolites of DEHP (MEOHP and MEHHP) in the same group of newborns and showed that inclusion of these metabolites in the analysis strengthened the association between the intensity of product use and exposure to DEHP (18). In our study, we investigated the excretion of DEHP metabolites in a group of high risk neonates sequentially, reporting not only the association with the intensity of exposure to DEHP containing products but also the duration of exposure and birthweight category of the patients.

The presence of DEHP in urinary samples may not always indicate exposure as this substance is a natural environmental contaminant. If plastic urine bags containing DEHP are used in collecting the specimens, this may result in overestimation of phthalate levels. To overcome this handicap, either non-DEHP containing materials such as cotton balls or glass tubes should be used in urine collection or secondary metabolites of DEHP (such as MEOHP or MEHHP) should be measured. We preferred to use glass tubes in urine collections and also measurement of secondary metabolites (MEOHP and MEHHP) in addition to DEHP. Blount et al (12) demonstrated that instead of the mother compound, measurement of phthalate monoesters (MEHP, MEOHP, and MEHHP) may prevent misdiagnosis of exposure. DEHP is rapidly hydrolyzed to MEHP, which may also be formed by a biotic processes such as hydrolysis, oxidation, and photolysis. MEHP is then oxidized to MEOHP and MEHHP. These secondary oxidized metabolites are not susceptible to contamination and are excreted in higher amounts than DEHP and MEHP. Calafat et al (16) reported that urinary concentrations of oxidized metabolites were higher and the concentrations of MEOHP and MEHHP were highly correlated. Due to contamination risks and low frequency of detection of DEHP and MEHP in urine samples, the use of MEOHP and MEHHP as biomarkers of phthalate exposure may be more appropriate. In our study, we showed that the urinary concentrations of MEOHP and MEHHP were highly correlated and that their concentrations were 18.4 and 24.5 times higher than that of MEHP, respectively. In addition to high concentrations, the high detection rate of MEHHP (81.4%) makes it a more suitable biomarker of phthalate exposure.

In order to determine exposure intensity, we measured the phthalate contents of unused medical devices in our NICU. Nasal cannulae had the highest amount of DEHP, followed by feeding tubes, endotracheal tubes, and umbilical catheters. Leakage of DEHP from these devices has been shown in previous studies. Takatori et al (22) simulated neonatal exposure to DEHP and MEHP from enteral nutrition products. They showed a 10-fold increase in the amount of phthalates in enteral nutrition fluid after transfer to PVC-containing enteral feeding bags and catheters. Chiellini et al (23) determined the phthalate contents of used and unused endotracheal tubes reporting the correlation between the leaching of phthalates and the duration of intubation in newborns. Latini and Avery (24) showed DEHP leakage and color change in endotracheal tubes after use in high risk newborns. They examined spectrocolorimetric changes in endotracheal tubes and determined significant color changes signifying *in vivo* degradation of DEHP-containing devices.

To our knowledge, sequential analysis of phthalate excretion in preterm infants during the course of their NICU stay has not been studied before. We showed that urinary concentrations of MEHHP and MEOHP increased in the first 4 weeks of life when intensive invasive procedures were performed. After this period, levels of DEHP metabolites started to decrease but did not disappear, indicating the persistence of exposure. MEHHP levels decreased in patients who had only feeding tubes after the second week of life, but stayed high in patients who continued to have additional invasive procedures. This result indicates the association between the intensity of exposure and excretion of DEHP metabolites as has been shown in other studies with the additional finding that duration of exposure is also important (17,18).

Concentration of phthalate metabolites may increase with immaturity. Zhang et al (25) showed that MEHP levels in cord blood, meconium and maternal serum samples of low birth weight infants were higher than those of normal birth weight infants. In two other studies, urinary levels of MEOHP and MEHHP in 6 extremely low birth weight infants were found to be approximately 10 times higher than those of 62 older children and adult patients (13,16). In our study we did not compare the levels of DEHP metabolites of the study group with those of term babies, but made a subgroup analysis of those babies with a birthweight less than 1000 g and ≥1000 g. MEHHP levels in the first urine samples of babies <1000 g were significantly higher than those ≥1000 g, although there was no significant difference in the intensity of invasive procedures between the groups. We speculated that this difference may be related to the level of immaturity or other undetermined antenatal exposure. We started to collect urine in the first 72 hours of admission to NICU. So increased levels of MEHHP in the first urine samples may be much more related with immaturity of our patients than maternal exposure. This is the limitation of our study. Future studies investigating maternal exposure, amniotic fluid and cord blood levels of DEHP metabolites in extremely low birth weight infants are needed to clarify this issue.

In conclusion, phthalates are endocrine-disruptors and normally should not be present in humans. There are only a few studies investigating DEHP exposure in preterm babies. The results of our study show that high amounts of oxidized

metabolites of DEHP in the urine samples of NICU patients can be detected and that this exposure is associated with the intensity and duration of invasive medical procedures. We showed that MEHHP was a more suitable biomarker of DEHP exposure. We do not know the long-term effects of this exposure during a very sensitive period of life, and we believe this is an issue which should be investigated in further studies. In the meantime, DEHP-free medical devices should be used for the patient population such as preterm infants which may be sensitive to the toxicity of phthalates.

Ethics

Ethics Committee Approval: The institutional review board of İstanbul University İstanbul Medical Faculty approved the study protocol (2010/716-208, 20.10.2010), Informed Consent: Informed consent was obtained from the parents.

Peer-review: Externally peer-reviewed.

Authorship Contributions

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Original Article

The Growth Characteristics of Patients with Noonan Syndrome: Results of Three Years of Growth Hormone Treatment: A Nationwide Multicenter Study

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Noonan syndrome is a multisystem disorders, and short stature is a frequent finding. There are no national data about the clinical and growth characteristics of Noonan syndrome patients until now.

WHAT THIS STUDY ADDS?

This study aimed to descript the characteristics of Noonan syndrome at a national level.

ABSTRACT

Objective: Noonan syndrome (NS) is a multisystem disorder, and short stature is its most striking manifestation. Optimal growth hormone (GH) treatment for NS is still controversial. In this study, using a nationwide registration system, we aimed to evaluate the growth characteristics and the clinical features of NS patients in Turkey and their growth response to GH treatment.

Methods: Children and adolescents with a diagnosis of NS were included inthe study. Laboratory assessment including standard GH stimulation test results were evaluated. Height increment of patients with or without GH treatment were analyzed after three years of therapy.

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Results: A total of 124 NS patients from different centers were entered in the web-based system. Short stature and typical face appearance were the most frequently encountered diagnostic features of our patients. Of the 84 patients who were followed long-term, 47 hadreceived recombinant human GH (rhGH). In this group of 47 patients, height standard deviation score (HSDS) increased from -3.62±1.14 to -2.85±0.96 after three years of therapy, indicating significant differences from the patients who did not receive GH treatment. *PTPN11* gene was analyzed in 61 patients, and 64% of these patients were found to have a mutation. HSDS at admission was similar in patients with or without *PTPN11* gene mutation.

Conclusion: A diagnosis of NS should be kept in mind in all patients with short stature showing systemic clinical findings. GH therapy is effective for improvement of short stature especially in the first two years of treatment. Further studies are needed for optimisation of GH therapy and evaluation of final height data in NS patients.

Keywords: Noonan syndrome, growth hormone treatment, growth

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Introduction

Noonan syndrome (NS) is a genetic, multisystem disorder with variable phenotype. The estimated prevalence of this syndrome was reported to vary between 1 in 1000 and 1 in 2500 live births (1,2). The main clinical characteristics of the syndrome consist of short stature, cardiovascular abnormalities (pulmonary valve stenosis, hypertrophic cardiomyopathy), cryptorchidism, and facial dysmorphology (hypertelorism, ptosis, low-set and posteriorly rotated ears, webbed neck) (3,4,5).

Short stature is a common manifestation of NS (6). Although birthweight and body length are usually normal in NS, pubertal growth is often delayed. At pubertal ages, short stature can be the most striking finding (3). Adult height in these patients is approximately 2 standard deviation (SD) below the mean for healthy adults (7). The causes of the growth disturbances in NS are multifactorial. Growth hormone (GH) deficiency, GH insensitivity, and neurosecretory dysfunction have been reported in these patients (3,8).

While the etiology of short stature is not definitely known, treatment of GH has been shown to improve growth rates (7). The response to GH therapy in NS can be affected by a number of factors such as dose of recombinant human GH (rhGH) and type of genetic mutation. The optimal GH treatment for NS is still controversial.

Genetic mutations associated with NS are involved in intracellular RAS/MAPK signal transduction pathway, leading to dysregulation (7). Until now, eight genes in the RAS/MAPK pathway, namely *PTPN11, SOS1, KRAS, NRAS, RAF1, BRAF, SHOC2*, and *CBL*, have been reported to cause NS. The *PTPN11* gene encodes the protein SHP2 which is responsible for controlling several developmental processes (3). Several studies have shown that almost half of NS patients had mutations in *PTPN11* gene (9,10).

Information about the clinical characteristics, especially in growth parameters of NS patients at national level is scarce. In this study, using a nationwide registration system, we aimed to evaluate the growth characteristics, clinical features, and response to GH treatment of NS patients in Turkey.

Methods

In this study, we retrospectively analysed the data of 124 children and adolescents with NS who were being followed in 20 centers in Turkey. Study approval was given by the Ankara University Ethics Committee. A nation-wide web based system was used for data collection which was realized between 15 May 2014 and 15 May 2015. A case recording form which covered demographic, clinical, and laboratory findings of patients was created and uploaded to the web site of FAVOR Web Registry System®. Data of patients aged between 0.2-18 years were entered in each center.

Children and adolescents with clinical (based on van der Burgt criteria) and/or genetic diagnosis of NS were included in the study. The following data on the patients' admission characteristics and clinical findings were collected: short stature, typical face dysmorphology, cardiac (pulmonary stenosis, hypertrophic cardiomyopathy, secundum atrial septal defect (ASD), electrocardiogram abnormalities, atrioventricular canal defect, mitral valve abnormalities, aortic coarctation), chest (pectus carinatum/excavatum, increased internipple distance, scoliosis), renal (unilateral kidney, pelvic dilatation), gastrointestinal (gastroesophageal reflux, recurrent vomiting, hepatomegaly, splenomegaly, feeding difficulties), hematologic (coagulopathy, thrombocytopenia, thrombocyte dysfunction), ocular (strabismus, refractive errors, amblyopia, nystagmus, cataract, fundal changes) anomalies, undescended testes, neuromotor problems (cognitive disorders, learning difficulties, mental retardation). The researchers were also asked to enter other clinical features that were not included in the questionnaire form to the system.

Clinical characteristics including birth weight, height, weight, height SD score (HSDS), body mass index (BMI), bone age at diagnosis were also recorded.

According to van der Burgt criteria, definitive NS was diagnosed by: 1) typical face dysmorphology plus one other major signs, or 2) suggestive face dysmorphology plus two other major or three minor signs. Major signs included 1) typical facial dysmorphology, 2) cardiac findings (pulmonary valve stenosis, hypertrophic obstructive cardiomyopathy, and/or echocardiography typical for NS), 3) height below 3rd centile, 4) chest wall deformities (pectus carinatum, pectus excavatum), 5) positive family history of a first-degree relative with a definite diagnosis of NS, 6) other findings (mental retardation, cryptorchidism, lymphatic dysplasia, etc.). Minor

signs included 1) suggestive facial dysmorphology, 2) cardiac defect other than major cardiac signs, 3) height below 10th centile, 4) broad thorax, 5) family history for first-degree relative with suggestive NS, 6) other findings (mental retardation, cryptorchidism, or lymphatic dysplasia) (11).

The questionnaire form also included the pathological genetic test results of the patient, if the genes *PTPN11, SOS1, RAF1, KRAS, NRAS*, etc. were analysed.

The researchers were also asked to enter to the system laboratory assessments including hormonal [thyroxine, thyroid-stimulating hormone (TSH), insulin-like growth factor 1 (IGF1), IGF-binding protein 3 (IGFBP3)] test results, GH stimulation tests, and bone age. If there were any other pathological laboratory findings, the participating centers were also asked to enter them. The assessment methods of serum IGF1, IGFBP3, and GH were answered optionally. Laboratory assessments included serum GH (ng/mL) levels measured by chemiluminescence method in all centers. Serum IGF1 and IGFBP3 were assayed by immunochemiluminescence (mostly) and immunoradiometric analysis (two cases).

All centers used two pharmacological GH stimulation tests for diagnosis of GH deficiency, in addition to clinical and laboratory characteristics. Sufficient GH response to GH stimulation tests were accepted as a peak GH level of >10 ng/mL. The participating centers were also asked if they had performed IGF generation tests in patients with low growth velocity.

All centers were also asked to enter to the system the yearly height increment of patients who were given GH treatment and those who were not. The follow-up form also included bone age increment and additional features developing during the follow-up.

Entering additional information not included in the questionnaire form was optional.

Statistical analyses were performed by using SPSS for Windows version 22.0 statistical software. Frequencies and percentages represented the descriptive statistics for categorical variables, and mean ± SD values were used for continuous variables. Student's t, chi-square, and Fisher exact tests were used. For evaluation of long-term growth parameters in groups, repeated measures ANOVA and Bonferroni test for pairwise comparisons were used.

Results

Baseline Characteristics

Data of a total of 124 patients with NS (84 males, 40 females) were entered to the web-based system. On admission, the mean age of patients was 8.36±4.5 years, HSDS was -3.13±1.31, and parentally adjusted height deficit was -2.25±1.73 (Table 1). Ninety eight of cases (79%) were prepubertal.

The most frequently seen clinical findings were short stature (88.7%) and typical facial features (88.7%). Cryptorchidism (uni- or bilateral) had been detected in 64% of male patients and cardiovascular anomalies in 62.8% of patients (Table 2).

Laboratory Characteristic

All NS patients were reported to be euthyroid on admission.

Genetic analysis of *PTPN11* gene could be performed in 61 cases, and 39 of these (64%) had mutations. The other mutations that caused NS could not be studied. One patient was reported to have *SOS1* gene mutation. Clinical characteristics of patient with and without *PTPN11* gene mutation were compared. The only difference detected between the groups was the percentage of typical facial dysmorphology. Patients with *PTPN11* gene mutation had 97.4% typical facial dysmorphology, but in patients without *PTPN11* gene mutation, this percentage was 78.9% (p=0.036). The remaining characteristics were similar between the two groups (Table 3).

Growth Hormone Treatment and Long Term Follow-Up

GH stimulation test was performed on 78 patients, and 50 of these showed suboptimal GH response (peak GH below 10 ng/mL). There were no statistically differences at admission in HSDS, serum IGF1 SD, and IGFBP3 SD between patients with GH deficiency and patients without GH deficiency.

Table 1. Characteristics of the patients at admission

	Mean ± SD	Median (minimum-maximum)
Chronological age (years)	8.36±4.5	9.08 (0.08-17.83)
Gestational age (weeks) (n=118)	38.46±2.38	39 (26-42)
Birth weight (g) (n=107)	2945±776	3000 (610-5000)
	-3.13±1.31	-3.1 (-7.79-1.29)
Mother's height (cm)	154.7±6.7	154.2 (136-174)
Father's height (cm)	168.23±7.54	168.6 (150-192)
Target height SDS	-0.81±1	-0.84 (-3.46-2.01)
Parentally adjusted height deficit (Target height SDS-height SDS)	-2.46±1.48	-2.22 (-6.5-2.48)
BMI (kg/m^2)	15.99±2.26	15.65 (10.4-27.1)
BMI SDS	-0.84±1.28	-0.79 (-5.5-1.95)
Bone age (years)	7.4±3.83	7.5 (0.5-18)
IGF1 SDS	-1.77±1.15	-1,67 (-4.17-1.23)
IGFBP3 SDS	-1.99±1.12	-1.95 (-4.72-1.05)

SD: standard deviation, BMI: body mass index, SDS: standard deviation score, IGF1: insulin-like growth factor 1, IGFBP3: insulin-like growth factor-binding protein 3

Table 2. Clinical findings of the patients		
Characteristics	**n**	**%**
Sex (Female/Male)	40/84	32.2/67.7
Diagnosed NS in family members	12	9.7
Pubertal state (prepubertal/pubertal)	98/26	79/21
Typical face dysmorphology	110	88.7
Cardiovascular abnormalities	78	62.9
Chest abnormalities	48	38.7
Neuromotor abnormalities	41	33.1
Ocular abnormalities	26	21
Hematological abnormalities	7	5.6
Renal abnormalities	8	6.5
Gastrointestinal abnormalities	19	15.3
Cryptorchidism*	54	64.2
NS: Noonan syndrome *In male patients		

Long-term growth follow-up data of 84 patients were evaluated. There were no differences in clinical characteristics on admission (typical face, cardiac, chest, eye, gastrointestinal, and other clinical findings) between these cases and those without follow-up. Among them, 47 patients had been receiving rhGH (mean dose= 0.25±0.05 mg/kg/week). Clinical and laboratory characteristics except HSDS were not different in patients receiving GH therapy as compared to patients not receiving GH therapy. GH therapy was introduced to shorter NS patients (Table 4).

HSDS increased from -3.62±1.14 to -2.85±0.96 after three years of therapy (Figure 1). Significant differences were observed compared to non-GH-treated patients for each year of therapy (p=0.02) (Table 5). Although bone age increment was evaluated as 1.3 years/year during the first year of therapy, there was no bone age acceleration during the second and third years of therapy.

Some additional findings developed in some patients during the follow-up period. Transient thrombocytopenia and splenomegaly developed in one case. In another case

Table 3. Characteristics of PTPN11 mutation-positive and negative cases			
Characteristics	**Mutation positive (n=39)**	**Mutation negative (n=21)**	**p-values**
Chronological age (years)	8.14±4.16	6.82±4.61	0.263
Sex (Female/Male)	12/27	9/13	0.423
Height SDS	-2.86±1.24	-2.77±1.36	0.175
BMI SDS	-1.07±1.25	-1.02±1.06	0.864
Birth weight (g)	3037±733	3051±610	0.562
Mother's height (cm)	154.23±7.88	154.85±5.63	0.75
Father's height (cm)	166.44±8.75	173.54±7.84	**0.01**
Target height SDS	-0.92±1.13	-0.4±0.96	0.129
Bone age (years)	7.42±4.05	7.46±4.44	0.979
IGF1 SDS	-1.69±0.77	-1.22±1.1	0.094
IGFBP3 SDS	-2.09±0.67	-1.49±1.11	0.085
BMI: body mass index, SDS: standard deviation score, IGF1: insulin-like growth factor 1, IGFBP3: insulin-like growth factor-binding protein 3			

Table 4. Characteristics of patients with long-term follow-up				
Characteristics (Mean ± SD)	**All patients with long-term follow-up**	**With GH treatment (n=47)**	**Without GH treatment (n=37)**	**p-values**
Chronological age (years)	9.31±4.57	9.8±3.4	8.66±3.8	0.16
Height SDS	-3.35±1.18	-3.62±1.14	-3±1.59	0.01
Target height SDS	-0,79±0,97	-0.88±1	-0.64±0.92	0.48
BMI	15.92±2.61	15.97±1.89	16.07±1.96	0.49
BMI SDS	-0.89±1.29	-0.82±1.31	-1.01±1.3	0.29
Bone age (years)	8.25±3.95	9.3±1.5	7.56±3.1	0.051
IGF1 SD	-1.88±1.11	-1.9±1.26	-1.6±1.05	**0.1**
IGFBP3 SD	-2.02±1.15	-2.1±1.4	-1.98±0.68	0.14
SD: standard deviation, GH: growth hormone, BMI: body mass index, SDS: standard deviation score, IGF1: insulin-like growth factor 1, IGFBP3: insulin-like growth factor-binding protein 3				

Table 5. Height increment of patients with Noonan syndrome			
	With GH treatment (n=47)	**Without GH treatment (n=37)**	**p-value**
Height increment at first-year follow-up	0.4±0.44	0.12±0.5	**0.08**
Total height increment at second-year follow-up	0.75±0.55	0.14±0.73	**0.001**
Total height increment at third-year follow-up	0.76±0.41	0.02±1.04	**0.009**
GH: growth hormone			

non-ossifying fibroma was diagnosed after beginning GH treatment. Insulin resistance was detected in one case which was not given GH treatment.

Only a small number of cases (n=5) reached their final height. Thus, this report does not cover any findings on final height.

Discussion

A total of 124 patients (84 males, 40 females) with NS had been registered to the system. All these patients were included in the study. Short stature and typical facial dysmorphism were the most frequent features of this group of patients.

The facial dysmorphology associated with NS such as hypertelorism, epicanthic folds and downward slanting palpebral fissures, low-set posteriorly rotated ears with a thick helix, high arched palate, micrognathia, and a short neck with excess nuchal skin and a low posterior hairline are the most recognizable features. The facial features were indeed the most frequently encountered findings in a high percentage (88.7%) of our patients. The diagnosis of NS is based on clinical features. Patients without typical facial features can easily go unnoticed and therefore, an awareness of suspicion of NS should be increased. In addition, it should be noted that the typical facial features decrease with age and patients with subtle phenotype cango undiagnosed, especially at older ages (11).

NS is one of the most common syndromic causes of congenital heart disease (1,5). In a large cohort study, cardiovascular disease was seen in 81% of NS patients (12). In our series, the proportion of cardiovascular was 62.9%. Pulmonary valve stenosis was detected in 45 patients and was the most frequent heart defect. Hypertrophic obstructive cardiomyopathy (HOCM) is not a frequent finding. van der Burgt (11) indicated that 20% of their NS patients had HOCM. The frequency of partial atrioventricular canal defect, secundum ASD was low in our cases. Anomalies on electrocardiogram such as wide QRS complexes, left axis deviation, giant Q waves were detected in 8 of our 124 patients.

Chest deformities which are characteristics for NS are pectus carinatum and pectus excavatum (11). We found that pectus carinatum occurred at almost twice the rate of pectus excavatum in our patients (32 vs. 17; 25% vs. 13.7%, respectively). The incidence of scoliosis was found lower than those reported in the literature (11,13).

Cryptorchidism is a common problem in male patients with NS. The incidence was reported to be as high as 80% (3,11). In our cases, the percentage of cryptorchidism was 64.2% which is relatively lower than the other series.

Feeding difficulties, especially during infancy, is another problem that can lead to calorie deficiency and growth failure. Gastroesophageal reflux is also a common problem in NS (1,5). The incidence of gastrointestinal problems was 15.3% in our series. In addition, rare gastrointestinal problems including splenomegaly, increase in liver enzyme levels, cholelithiasis, cleft lip and palate were also reported.

Neuromotor disorders, especially mental retardation and learning difficulties, were observed in an important percentage of our patients (Table 1). Mental retardation is usually mild, and this finding is in agreement with the experience of others (11).

Strabismus, refractory errors, amblyopia were the most prominent eye features of our patients. Two of our cases had retinitis pigmentosa.

Hematological and renal system involvements were the least frequently reported findings in our series.

Overall, the systemic findings in our series of patients were not strictly similar to previously reported data. There were some differences either in percentage or in severity of clinical findings. These differences can possibly be attributed to factors such as population characteristics, number of evaluated cases, variation in genetic mutations. We collected data from only pediatric endocrinology clinics. Patient characteristics could be different in those admitted to cardiology or other clinics.

Genetics

Genetic heterogeneity is a well-known characteristic of NS. There are eight genes in the RAS/MAPK signaling pathway causing NS (3,5). In our series, 61 patients were analysed for *PTPN11* gene mutation. The other genes, except for one patient with SOS1 mutation, were not analysed. The rate of *PTPN11* gene mutation was 64% of PTPN11 in the patients who underwent genetic analysis.

In the literature, PTPN11-which encodes the protein SHP2- is the most frequently encountered gene associated with NS (3,5,9). Similar to our series, *PTPN11* gene mutation was reported in almost half of the NS patients in other

studies (2,3,10). *SOS1* and *RAF1* genes each contribute by 10% to genetic causes of NS, while *KRAS* and *NRAS* gene mutations have been detected only in a very small number of cases (3).

There are reports that indicate some differences in NS patients carrying *PTPN11* gene mutation from the others (3,9), while others have reported that there were no clinical and laboratory differences between the groups (2,14). Binder et al (10) reported that mutation in *PTPN11* gene is associated with mild GH resistance in NS. They emphasized that the mean change in HSDS after one year of GH therapy was lower in mutation-positive patients than mutation-negative patients. In addition, pulmonary stenosis and ASD were most frequently seen in *PTPN11* gene mutation-positive patients.

In NS patients with *PTPN11* gene mutations, bleeding diathesis, juvenile myelomonocytic leukemia, cardiac defects, typical facies, cryptorchidism, and short stature were also reported to be more common than in patients without *PTPN11* gene mutations (3,9,10).

In our series, typical facial features were more prominent in patients with *PTPN11* gene mutations, and this was the only difference between patients with and without *PTPN11* gene mutation. Growth parameters were similar in mutation-negative and mutation-positive patients.

Growth Hormone Therapy

The causes of growth deficiency are heterogeneous in NS. GH deficiency, neurosecretory dysfunction, GH insensitivity were suggested factors (8,10,15). In our series, GH stimulation tests were done in 78 patients, and 50 of these (64%) showed suboptimal responses. IGF generation test was performed in two patients, and the results were found to be in line with low GH secretion. Interestingly, the degree of short stature was not different between patients with normal and low GH responses to stimulation tests. Serum IGF1 and IGFBP3 levels were also similar in these two groups of patients.

Some authors suggested that patients with *PTPN11* gene mutations often have short stature, low IGF1, and normal or high GH serum levels (10,16). The effect of *PTPN11* gene mutations on growth retardation could not be demonstrated in this present study. There were no differences in HSDS between patients with *PTPN11* gene mutation and those without mutation. There were also no differences according to serum IGF1 and IGFBP3 levels.

An important fraction of NS patients in this study (n=84) were followed for three years. Forty seven of these were given GH treatment, while 37 were not. HSDS values at admission were lower in GH-treated NS patients, and this finding was the only difference between the GH-treated patients and the non-GH-treated group.

HSDS of NS patients significantly increased during GH treatment, and a positive effect on growth was observed. HSDS increasedby 0.4±0.44 SD during the first year of treatment, and total increments were 0.75±0.55 SD and 0.76±0.41 SD at the end of the second and third years of treatment. HSDS of patients without GH therapy did not change during follow-up (Figure 1).

Despite a positive increment in HSDS noted especially during the first year of GH therapy, this was below 0.5 SD height, a figure which is a relevant indicator of good response to GH therapy (17).

Similar to our study, short-term studies with GH treatment reported an increase of mean HSDS and/or height velocity. After one year of GH therapy, the increment of HSDS was found to be between 0.3 and 0.8 SD. It was also reported that the changes in HSDS and/or height velocity in NS were similar to those observed in Turner syndrome patients (6,18,19,20,21,22). In these studies, the doses of GH were also similar. In our patients, the doses of rhGH varied between 0.2 to 0.35 mg/kg/week, with a mean dose of 0.25 mg/kg/week, which is lower than the offered doses in NS patients. GH resistance in some NS cases would require higher doses of GH.

All these findings indicate that in Turkey, there is still a need to optimise GH therapy in NS patients and most importantly, individualisation of treatment can lead to optimisation of therapy.

No serious side effects related to GH treatment were detected in our series. Transient thrombocytopenia and splenomegaly in one case, non-ossifying fibroma in another case were seen while receiving GH treatment. Insulin resistance was reported in one case who was not receiving GH therapy.

We carefully evaluated the possibility of bone age acceleration in GH-treated NS patients. With GH therapy, an acceleration of bone age was reported in most cases. Bone age has been reported to advance by 1.1-1.2 years/

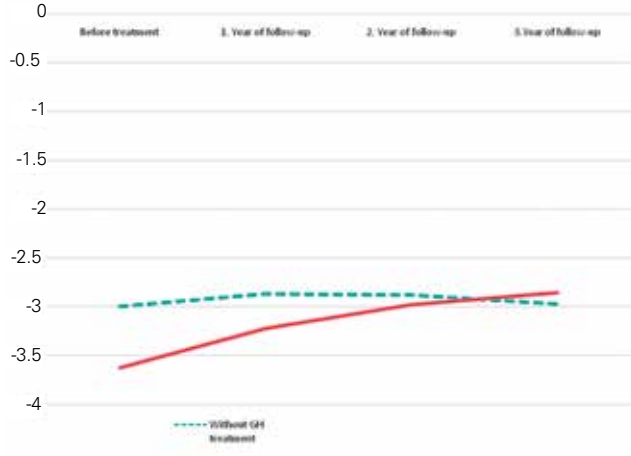

Figure 1. Changes in height standard deviation of patients during follow-up.
GH: growth hormone

year during GH therapy (15,21,23). We observed a similar trend, especially during the first year of therapy. Actually, NS patients generally have a delayed bone age at the start of GH treatment. It is accepted that the acceleration of bone age reflects normalisation (7).

There are reports indicating that final height of NS patients receiving GH showed a gain of SDS ranging from 0.6 to 1.7 (6,24). We have only a few patients who reached their adult height (n=5) and therefore we cannot report any conclusions.

There are some limitations of our study. First of all, the data was web based and, being from different centers, was very heterogeneous in some clinical or laboratory characteristics. Indications for and dose of GH therapy were not uniform. In addition, the phenotypic, laboratory, and molecular characteristics of NS patients were not similar. The systemic features of NS were more frequently reported in some centers. Response to GH therapy also showed variations, indicating a need for individualisation of GH therapy for optimal response.

In conclusion, in this study, we attempted to demonstrate the clinical and biochemical characteristics of NS patients at a national level. Our data support the findings in previous reports and indicate that in NS patients, GH therapy is useful to improve height deficit, at least during the first two years of therapy. We suggest that GH therapy optimisation is needed for NS patients. Further randomized, observational studies are necessary on patients receiving different GH doses. Also, genetic analysis of patients without *PTPN11* gene mutation will provide additional information on NS patients.

Ethics

Ethics Committee Approval: Ankara University Ethical Committee 2014, approval number: 10.02.2014, 03-84-14, Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Zeynep Şıklar and Merih Berberoğlu, Design: Zeynep Şıklar and Merih Berberoğlu, Data Collection or Processing: Zeynep Şıklar, Mikayir Genens, Şükran Poyrazoğlu, Firdevs Baş, Feyza Darendeliler, Rüveyde Bundak, Zehra Aycan, Şenay Savaş Erdeve, Semra Çetinkaya, Ayla Güven, Saygın Abalı, Zeynep Atay, Serap Turan, Cengiz Kara, Gülay Can Yılmaz, Nesibe Akyürek, Ayhan Abacı, Gamze Çelmeli, Erkan Sarı, Semih Bolu, Hüseyin Anıl Korkmaz, Enver Şimşek, Gönül Çatlı, Muammer Büyükinan, Atilla Çayır, Olcay Evliyaoğlu, Pınar İşgüven, Tolga Özgen, Nihal Hatipoğlu, Atilla Halil Elhan, Merih Berberoğlu, Analysis or Interpretation: Zeynep Şıklar and Merih Berberoğlu, Literature Search: Zeynep Şıklar, Writing: Zeynep Şıklar.

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Original Article

Soluble Endoglin Level Increase Occurs Prior to Development of Subclinical Structural Vascular Alterations in Diabetic Adolescents

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ABSTRACT

Objective: Soluble endoglin (S-endoglin) has been implicated as a potential marker of endothelial dysfunction (ED) and was reported to be elevated in diabetic adults, correlating with the severity of diabetic vasculopathy. However, circulating S-endoglin and its association with other markers of ED have not been formerly analyzed in the first decade of diabetes onset in adolescents with type 1 diabetes mellitus (T1DM).

Methods: Fifty-eight adolescents with moderately/poorly controlled T1DM were included in this study and twenty-nine healthy adolescents served as controls. The diabetic group was divided into two groups based on the presence of microalbuminuria, as the microalbuminuria group (n=15) and the normoalbuminuria group (n=43). Functional vascular alterations were evaluated by measuring serum S-endoglin and plasma nitric oxide (NO) concentrations, the flow-mediated dilatation (FMD) of the brachial artery. Carotid intima media thickness (CIMT) was measured for evaluation of structural vascular alterations.

Results: The S-endoglin and NO levels of both microalbuminuria and normoalbuminuria groups were higher than those of the control group (for S-endoglin, p=0.047 and p<0.001; for NO, p=0.004 and p=0.006, respectively). The FMD percent was lower in the microalbuminuria group compared to the normoalbuminuria and control groups (p=0.036 and p=0.020, respectively). There were negative correlations between S-endoglin concentration and FMD percent (r=-0.213, p=0.051) and between serum S-endoglin concentration and albumin excretion rate (r=-0.361, p=0.005). No significant differences were found in CIMT among any of the groups (p=0.443).

Conclusion: In adolescents with T1DM, S-endoglin concentrations might increase in parallel to the deterioration in endothelial function before subclinical structural vascular alterations become evident.

Keywords: Type 1 diabetes mellitus, subclinical atherosclerosis, soluble endoglin, adolescents

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Soluble endoglin molecule has been suggested as a potential biomarker of endothelial dysfunction since its concentration was found elevated in several diseases with vascular involvement. Recently, concentration of this molecule has been found to be increased in diabetic adults, correlating with the severity of diabetic vascular insult.

WHAT THIS STUDY ADDS?

We analyzed soluble endoglin concentration and its relation with other suggested markers of endothelial dysfunction in the first decade of diabetes onset in adolescents with type 1 diabetes mellitus.

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Introduction

Hyperglycemia causes subclinical functional and structural vascular alterations associated with premature atherosclerosis even in childhood type 1 diabetes mellitus (T1DM). It precipitates the early emergence of endothelial dysfunction (ED) as a subclinical functional vascular alteration representing the initial step toward the atherosclerotic process that promotes the development of cardiovascular diseases (CVDs) and also of microvascular complications in patients with T1DM. Several biomarkers and also radiological methods including measurement of flow-mediated dilatation (FMD) of the brachial artery and carotid intima media thickness (CIMT) have been used to assess diabetes-related early vascular alterations. Changes in the amount and/or bioavailability of nitric oxide (NO) molecule, the major agent that contributes to the anti atherosclerotic effects of endothelium, constitute one of the early findings of ED (1,2,3,4).

Another molecule which has been implicated in the regulation of endothelial function is endoglin, a 180 Kda homodimeric integral membrane glycoprotein serving as a receptor for the transforming growth factor-β (TGF-β) superfamily (5,6,7,8). Demonstration of increased endoglin expression in atherosclerotic plaques suggested the participation of endoglin in the atherosclerotic process (8). Furthermore, a soluble form of endoglin (S-endoglin), generated by the cleavage of the extracellular domain of the entire endoglin molecule, has also been suggested as a marker of ED and reported to be increased in the serum of patients with preeclampsia, hypercholesterolemia, and atherosclerosis (9,10,11,12). In addition, the circulating level of S-endoglin has also been shown to be elevated in patients with type 2 diabetes mellitus and considered as an indicator of diabetes-related vascular pathologies (13). However, it is not known whether circulating S-endoglin level changes in the first decade of T1DM onset is a potential early marker of ED in adolescents with T1DM.

A widely used and reliable radiological method to measure endothelial function in patients with T1DM is FMD of the brachial artery (14,15). Numerous studies have consistently reported that children with T1DM have decreased FMD percentage relative to healthy controls (15,16). Furthermore, some studies reported increased CIMT, as a next stage, representing the occurrence of subclinical structural vascular alteration in young subjects with T1DM (16,17).

Circulating S-endoglin level was found to be elevated in diabetic adults correlating with the severity of diabetic vascular changes that suggested S-endoglin as a potential marker of ED. However, circulating S-endoglin level and its relation with other suggested markers of ED have not been investigated in the first decade of T1DM onset in diabetic adolescents. Therefore, in the present study, we evaluated subclinical vascular alterations radiologically by ultrasonographic measurement of FMD and CIMT, and biochemically by measurement of plasma NO level along with serum S-endoglin level in adolescents with T1DM.

Methods

This cross-sectional study was performed during the period September 2013 to February 2014 and included 58 adolescents with T1DM followed in Gazi University Faculty of Medicine Hospital, Pediatric Endocrinology Clinic and 29 group-matched healthy controls. Data on age, gender, duration of diabetes, insulin regimen, and daily requirement for insulin and mean annual glycated hemoglobin (HbA_{1C}) levels were collected from the medical records. Mean HbA_{1C} levels of the follow-up period (f-HbA_{1C}) and the preceding year (py-HbA_{1C}) were calculated. HbA_{1C} levels at the time of T1DM diagnosis were excluded while calculating f-HbA_{1C} levels. Diabetic adolescents (n=58) were divided into two groups based on the presence of persistent microalbuminuria, as the microalbuminuria group (n=15) and the normoalbuminuria group (n=43). Persistent microalbuminuria was defined as a urinary albumin excretion rate (AER) between 30-300 mg/dL in at least two of three 24-hour urine samples over a 3-month period (18). None of the diabetic adolescents were receiving any treatment with other drugs except insulin.

All participants were subjected to physical examination. Height was measured to the nearest centimeter using a Harpenden stadiometer (Holtain Instruments Ltd, UK). Weight was measured unclothed to the nearest 0.1 kg using a calibrated balance scale. Body mass index (BMI) was calculated using the weight (kg)/height (m^2) equation. Standard deviation scores (SDS) for weight, height, and BMI were calculated using the reference values for Turkish children (19). Measurements of blood pressure were performed in all cases after a period of resting and were repeated 3 times with 10-minute intervals. Subjects with systolic and/or diastolic blood pressure above the 95th percentile were accepted as hypertensive. Pubertal status of each case was defined according to Tanner criteria. All subjects were nonsmokers and were normotensive, had normal plasma lipids, liver and renal functions, plasma electrolyte levels, and a normal blood count. Exclusion criteria of the patients with T1DM included smoking, dyslipidemia, hypertension, and presence of a chronic disease other than T1DM. The healthy control adolescents included in this study were volunteers in Gazi University Faculty of Medicine Hospital. Their inclusion criteria were good health, no known history of chronic disease, and no medications which might influence cardiovascular function, glucose, or lipid metabolism. The study protocol was approved by Gazi University Faculty of Medicine Clinical Trial Ethics Committee. Informed consent and assent were obtained from all subjects and their parents.

Peripheral venous blood samples were obtained to determine fasting plasma glucose (FPG), lipids [total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL), triglycerides], blood urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), NO, and serum S-endoglin concentrations after an overnight 12-hour fast. AER was measured in 24-hour urine samples. These parameters were evaluated on a blind basis in Gazi University Faculty of Medicine Hospital Biochemistry Laboratory using standard automatized techniques. Plasma glucose concentrations were measured by glucose oxidase reaction. BUN, creatinine, AST, ALT, and lipid concentrations were measured by spectrophotometric methods, and albumin concentrations in 24-hour urine samples were measured by immunoturbidimetry on an autoanalyzer (Beckman Coulter, La Brea, CA, USA).

Subclinical functional vascular alterations were evaluated by measuring plasma NO and serum S-endoglin concentrations and FMD of the brachial artery, whereas subclinical structural vascular alteration was evaluated by measuring CIMT. Serum S-endoglin concentrations were measured by using an enzyme-linked immunosorbent assay method (Human Endoglin; R&D Systems, Minneapolis, MN, USA). Plasma NO concentrations were determined using the Griess reaction by measuring combined oxidation products of NO, plasma nitrite (NO2), and nitrate (NO3) after reduction with nitrate reductase in a colorimetric assay (Cayman Inc., Ann Arbor, Michigan, USA). Before initiation of the assay to prepare sample solutions, hemoglobin and proteins were removed using a membrane filter (Amicon Ultra 10 Kda Ultracel; Millipore, Darmstadt, Germany). The intra- and inter-assay coefficients of variation for S-endoglin were 3% and 6.3% and for NO, 2.6% and 4.2%, respectively.

Flow-Mediated Dilatation Measurements

Patients were instructed to avoid any food or drink containing caffeine before the procedures as such substances may interfere with endothelial functions. After the patients had rested for ten minutes in supine position, the brachial artery was located in the antecubital fossa and its basal diameter was measured with a 12 MHz high-resolution linear probe. Following the optimal basal measurement, systolic blood pressure was increased to 250 mmHg with the instrument of the cuff placed above the measurement site, and the ischemia and shear stress were sustained for 5 minutes. The cuff was then deflated and sequential re-measurements of the brachial artery diameter were done with 30-second intervals for 2 minutes. The peak brachial artery diameter that was measured after the deflation of the cuff was recorded. Finally, the percentage flow-mediated dilatation index (FMD%) was calculated by dividing the maximum arterial diameter change by the basal arterial diameter (14,20).

Carotid Intima-Media Thickness Measurements

After a period of 10-minute rest, CIMT was measured using the 12 MHz linear probe while the patients were in supine position with slight extension of the head towards the opposite of the carotid artery of interest. An optimal longitudinal, 2-dimensional image of the distal common carotid artery was frozen on screen. The CIMT was determined by taking the mean of all three measurements (21).

Statistical Analysis

After entering the data using the Statistical Package for the Social Sciences (SPSS) Version 18.0 software (SPSS Inc., Chicago, IL, USA), the analysis of the results were performed using percentage distribution for qualitative data and median interquartile range (IQR) or mean (standard deviation) for quantitative data. The statistical tests used were the Shapiro-Wilks test for normality, the chi-square test for qualitative data comparison of groups, and the independent samples test, Mann-Whitney U-test, one way analysis of variance (ANOVA), and Kruskal-Wallis test for quantitative data comparison of groups, as appropriate. The correlation between quantitative data was calculated by Spearman rank correlation. A p-value less than 0.05 was considered statistically significant.

Results

Eighty-seven adolescents (40 female) were included in the study. Except for FPG concentrations, there were no significant differences for demographic and metabolic characteristics among the groups ($p>0.05$ for all, except FPG). No statistically significant difference was found for systolic and diastolic pressures between the groups ($p=0.483$ and $p=0.625$, respectively). None of the participants had hypertension. Neither mean f-HbA_{1C} nor mean py-HbA_{1C} levels significantly differed between the microalbuminuria and normoalbuminuria groups (Table 1).

Serum S-endoglin concentration significantly differed among the groups ($p<0.001$). Both normoalbuminuria and microalbuminuria groups had significantly higher S-endoglin concentrations [2.50 (2.19-3.09) ng/mL and 2.21 (1.91-2.90) ng/mL, respectively] compared to the control group [1.97 (1.72-2.23) ng/mL] ($p<0.001$ and $p=0.047$, respectively) (Figure 1a). S-endoglin concentration was higher in the normoalbuminuria group compared to the microalbuminuria group but did not reach statistical significance ($p=0.108$).

The difference in plasma NO concentration was significant among the groups ($p=0.005$). NO concentrations of both microalbuminuria and normoalbuminuria groups [41.8 (37.7-51.2) µmol/L and 42.5 (30.1-54.9) µmol/L, respectively] were significantly higher compared to the control group [30.8 (25.7-40.4) µmol/L] ($p=0.004$ and $p=0.006$, respectively) (Figure 1b).

Table 1. Demographic and metabolic characteristics of the study subjects

	Microalbuminuria group (n=15)	Normoalbuminuria group (n=43)	Control group (n=29)	p-value
Demographic parameters				
Sex (% Female)	53.3	65.3	51.7	0.714
Age (years)	16.3±2.17	15.14±1.55	15.03±1.97	0.061
Height z-score	0.37±0.53	-0.08±0.91	0.01±0.91	0.059
BMI z-score	0.62±0.53	0.57±0.76	0.26±0.69	0.142
Metabolic parameters				
FPG (mg/dL)	214±95.3	199.4±79.2	87.8±7.1	<0.001
Cholesterol (mg/dL)	161.6±32.7	172.5±37.5	155.3±23.4	0.052
HDL-cholesterol (mg/dL)	49.51±6.60	51.6±10.20	49.4±8.02	0.690
LDL-cholesterol (mg/dL)	93.1±29.3	100.9±33.4	91.0±22.5	0.081
Triglycerides (mg/dL)	75 (63-109)	90 (65-120)	68 (58-91)	0.054
AST (IU/L)	19 (17-25)	20 (16-25)	22 (19-28)	0.108
ALT (IU/L)	16 (13-19)	14 (12-21)	12 (12-17)	0.266
BUN (mg/dL)	12.2±2.5	11.4±2.5	10.1±2.5	0.060
Creatinine (mg/dL)	0.59±0.10	0.57±0.10	0.56±0.10	0.728
Diabetes duration (years)	8 (5-11)	5.5 (3.75-8.5)	-	*0.058
f-HbA_{1c} (%)	9.14±1.71	8.86±1.72	-	*0.591
py-HbA_{1c} (%)	9.59±2.24	9.30±2.23	-	*0.365
AER (mg/day)	53 (36.0-80.0)	5.8 (9.8-13.7)	-	†<0.001

Data are presented as means ± SD except for non-normally distributed triglycerides, aspartate aminotransferases, alanine aminotransferases, diabetes duration, and albumin excretion rate that are presented as median (25^{th}-75^{th} percentiles). BMI: body mass index, FPG: fasting plasma glucose, LDL: low density lipoprotein, HDL: high density lipoprotein, AST: aspartate aminotransferase, ALT: alanine aminotransferase, BUN: blood urea nitrogen, f-HbA_{1C}: mean glycated hemoglobin since the diagnosis of diabetes, py-HbA_{1C}: mean glycated hemoglobin of the preceding year, AER: albumin excretion rate

*p>0.05 microalbuminuria group vs. normoalbuminuria group

†p<0.05 microalbuminuria group vs. normoalbuminuria group

No significant difference was detected in NO level between the microalbuminuria and normoalbuminuria groups (p=0.703).

FMD% significantly differed among the groups (p=0.032). The microalbuminuria group had a significantly lower FMD% (7.53±3.29%) compared to the control and normoalbuminuria groups (10.9±4.01% and 9.93±3.51%, respectively) (p=0.020 and p=0.036, respectively) (Figure 1c). The FMD% was lower in the normoalbuminuria group than in the control group, but the difference was not statistically significant (p=0.306). The CIMT was slightly higher in the microalbuminuria group [0.44 (0.42-0.55) mm] compared to the normoalbuminuria group [0.43 (0.40-0.48) mm] and the control group [0.43 (0.37-0.48) mm], but this difference did not reach statistical significance (p=0.443) (Figure 1d). There were no gender differences in S-endoglin, NO, FMD, and CIMT measurements in the diabetic group or in the overall study population (p>0.05 for all).

Significant negative correlations were found between serum S-endoglin concentration and AER (r=-0.361, p=0.005) as well as between S-endoglin concentration and FMD% (r=-0.213, p=0.051) (Figure 2). There was a weak positive association between S-endoglin and NO concentrations (r=0.203, p=0.059). Except for these, there were no associations between the parameters evaluated in this study (p>0.05 for all).

Discussion

In the first decade of T1DM onset, we found significantly increased plasma NO concentrations as well as increased S-endoglin concentrations in both microalbuminuria and normoalbuminuria groups relative to the control group. On the other hand, there were no significant differences in CIMT among any of the groups. Furthermore, we detected a weak negative association between S-endoglin concentration and FMD% and a weak positive association between S-endoglin and NO concentrations. In the light of these data, we suggested that in adolescents with T1DM, S-endoglin concentrations might increase in parallel to the deterioration in endothelial function before subclinical structural vascular alterations became evident. Moreover, considering the inverse association between circulating S-endoglin and AER and the presence of slightly lower S-endoglin concentration in the microalbuminuria group compared to the normoalbuminuria group, we speculate that

exposure to T1DM initially may give rise to an increase in serum S-endoglin concentrations; however, with the development of microalbuminuria, a relative decline in circulating S-endoglin concentrations might be observed. All these findings suggest that increased circulating S-endoglin may be an early indicator of diabetes-related functional vascular alterations and even might precede the development of microalbuminuria, before the manifestation of subclinical structural vascular alterations in adolescents with T1DM.

Changes in the bioavailability of NO molecule have been reported as an indicator of ED in diabetic vasculopathy (22). However, while increased levels of NO production were detected in early diabetes, as the duration of diabetes increases, reduced blood NO concentrations were measured in diabetic patients (3,4,23,24,25). Accordingly, we found significantly higher plasma NO concentrations in both microalbuminuria and normoalbuminuria groups relative to the control group in the first decade of T1DM onset. Although the reasons are not fully known, induction of inducible NO synthase synthesis and upregulation of NO production as a response to its reduced bioavailability due to enhanced free radical formation are suggested mechanisms for increased NO production in early diabetes (26,27).

In a considerable number of studies, it has been reported that impaired FMD, as a reliable indicator of ED, may become evident even a few years after the onset of T1DM (15,16). We found significantly reduced FMD in the microalbuminuria group compared to both normoalbuminuria and control groups, whereas such a significant difference was not detected between the normoalbuminuria and control groups. It was widely assumed that FMD was a direct indicator of NO bioavailability in the endothelium. However, considerable evidence showed that FMD response in the conduit arteries is not merely NO-dependent (28,29). Although controversy exists, certain well-designed studies reported that substances used to block NO synthesis were not able to deteriorate the FMD response (30). Moreover, it has been demonstrated that endothelial NO synthase knockout mice were still capable of dilating their arteries as a response to shear stress due to release of additional vasodilatory molecules, such as prostacyclin (PGI2) and endothelial-derived hyperpolarizing factor which may contribute to FMD (31). On the other hand, impact of these molecules and NO on FMD may vary according to shear stress creation technique, vascular bed, and diseased states (28,29). In line with these data, in our study, despite the presence of more impaired FMD in the microalbuminuria group relative to the normoalbuminuria group, plasma NO concentrations did not significantly differ between the microalbuminuria and normoalbuminuria groups, and furthermore, we did not find a correlation between plasma NO concentration and FMD%. According to the above-mentioned findings, it may be suggested that circulating NO level alone, as an indicator of ED, may not be sufficient to predict the severity of ED in adolescents with T1DM.

Recently, S-endoglin has been implicated as a potential marker of ED (6). Hypoxia and oxidative stress are considered triggers of S-endoglin release, which, in turn, inhibits the anti-atherogenic effects induced by TGF-β (32,33). Although a more recent study has demonstrated that increased S-endoglin level per se is not capable of inducing ED in an animal model, the authors indicated that their finding does not rule out the possibility that S-endoglin might contribute to alteration of endothelial function in the presence of other risk factors related to CVDs (34). Increased circulating concentrations of S-endoglin

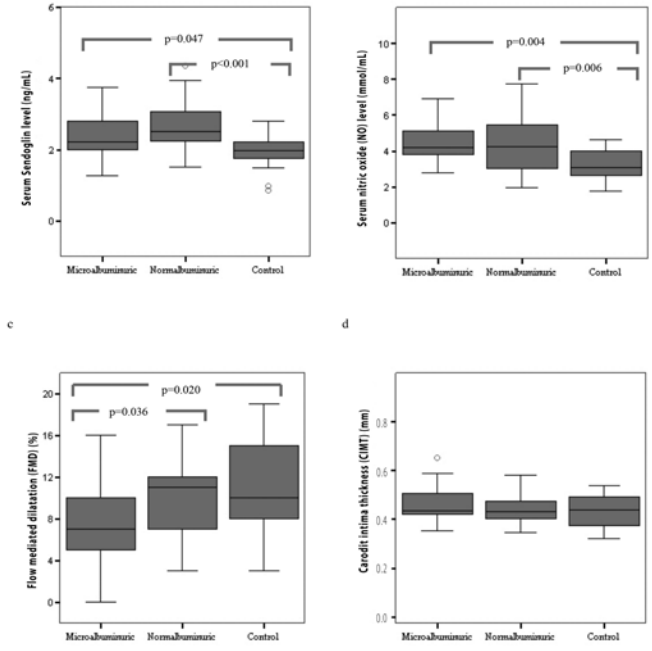

Figure 1. Comparison of NO, S-endoglin, FMD, and CIMT measurements among groups. Kruskal-Wallis test over all groups for NO p<0.05 and for CIMT p>0.05. One way ANOVA test over all groups for S-endoglin and FMD p<0.05. p-values of pairwise comparisons (Mann-Whitney U test and independent samples t-test) are shown in the diagram. NO: nitric oxide, S-endoglin: soluble endoglin, FMD: flow-mediated dilatation, CIMT: carotid intima-media thickness, ANOVA: analysis of variance

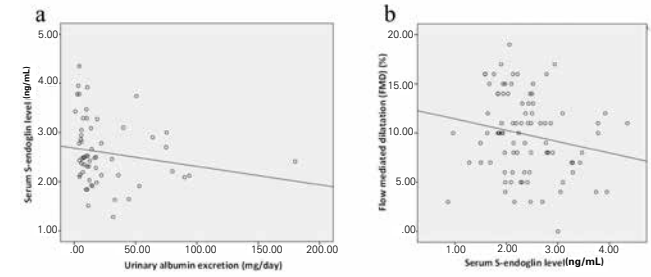

Figure 2. Relationship of serum S-endoglin level with urinary albumin excretion (Figure 2a; r=-0.361) and flow-mediated dilatation % (Figure 2b; r=-0.213). S-endoglin: soluble endoglin

were reported to be associated with vascular damage in several disease states including preeclampsia, hypercholesterolemia, and atherosclerosis (10,11,12). A similar relationship between increased serum S-endoglin concentrations and diabetes-related vascular disorders and a positive association between circulating S-endoglin and ED have also been demonstrated in an adult study from Spain (13). In addition, glucagon-like peptide-1 has been shown to reduce plasma S-endoglin levels and oxidative stress in patients with T1DM, presumably due to its intracellular antioxidant activity (35). Our study is the first to assess serum S-endoglin concentrations as a potential early marker of ED representing subclinical vascular alterations in a young cohort with T1DM. It is known that children with T1DM may develop ED within the first decade after its onset and that diabetes-related structural vascular alterations occur after the development of diabetes-related functional vascular alterations. In our study, there was no significant difference in CIMT among the three groups, however, as was also true for NO concentrations, significantly increased circulating S-endoglin concentrations were measured in both microalbuminuria and normoalbuminuria groups relative to the control group. Moreover, we detected a weak linear correlation between S-endoglin and NO concentrations as well as a weak inverse correlation between S-endoglin and FMD%; these are findings which might favor the probable association of S-endoglin with ED in T1DM. In the light of these data, we suggest that in adolescents with T1DM, S-endoglin concentrations might increase in parallel to the deterioration in endothelial function prior to the appearance of subclinical structural vascular alterations. However, long-term prospective studies investigating the association of S-endoglin with other indicators of endothelial function are needed to confirm these findings.

Li et al (36) found S-endoglin and TGF-β1 concentrations of patients with severe coronary atherosclerosis significantly lower than those of patients with mild coronary atherosclerosis and those of healthy controls. They proposed that in the early stages of atherosclerosis, circulating concentration of S-endoglin increases due to damage of endothelial cells, but with the progression of the atherosclerotic process, S-endoglin concentration decreases due to elevated levels of S-endoglin/TGF-β1 complexes in blood serum (36). Likewise, with the progression of ED during the course of T1DM, alterations in serum S-endoglin concentration may be observed. S-endoglin may be important in only certain stages of diabetes-related vascular insult. The circulating concentration of S-endoglin may decrease over time and this decrease may be due to decreased production or enhanced complex formation with other yet unidentified substances in the circulation. In our study, the highest S-endoglin concentration was found in the normoalbuminuria group. As compared to the normoalbuminuria group, the microalbuminuria group had an insignificantly lower S-endoglin concentration. However, this latter group had significantly reduced FMD which confirms the presence of more evident ED in the microalbuminuria group. As known, microalbuminuria is an indicator of generalized ED and is regarded as a common pathway of injury to both renal and systemic vascular beds (37). Of note, we detected a significant inverse association between AER and serum S-endoglin concentration. According to the above-mentioned results, it may be speculated that in adolescents with T1DM, exposure to a diabetic milieu might initially lead to an increase in circulating concentrations of S-endoglin, but, with the development of microalbuminuria, a relative decrease may be observed in these levels.

Our study has several limitations. First, the small sample size of the cohort might have undermined the power of this study while making conclusions due to the alterations in markers used in the evaluation of subclinical atherosclerosis. Absence of certain associations could also be related to this limitation. Second, our study design was a cross-sectional one which may not provide definite information about cause-and-effect relationships. Therefore, the associations of S-endoglin with ED and the long-term micro- and macrovascular complications of T1DM need to be analyzed prospectively.

Our findings suggest that within the first decade of T1DM onset, circulating concentrations of S-endoglin may increase before an increase in CIMT and with the rise of AER above a definite critical level which might be closer to the lower range of microalbuminuria; a comparative decrease in the circulating S-endoglin level may be observed in adolescents with T1DM. However, long-term prospective studies measuring S-endoglin concentrations with recognized indicators of ED are needed to better elucidate the relationship of S-endoglin with atherosclerotic process and microvascular complications of T1DM.

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Ethics

Ethics Committee Approval: This study was approved by the Scientific Review Committee of Gazi University Faculty of Medicine (approval number: 25901600/5447), Informed Consent: It was taken.

Peer-review: Externally peer-reviewed.

Authorship contributions

Concept: Hamdi Cihan Emeksiz, Aysun Bideci, Design: Hamdi Cihan Emeksiz, Aysun Bideci, Data Collection or processing: Hamdi Cihan Emeksiz, Çağrı Damar, Betül Derinkuyu, Nurullah Çelik, Esra Döğer, Özge Yüce, Analysis or

Interpretation: Hamdi Cihan Emeksiz, Aysun Bideci, Mahmut Orhun Çamurdan, Peyami Cinaz, Mehmet Cüneyt Özmen, Literature Search: Hamdi Cihan Emeksiz, Aysun Bideci, Writing: Hamdi Cihan Emeksiz, Aysun Bideci.

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Original Article

Adding Multiple Adipokines into the Model do not Improve Weight Gain Prediction by Leptin Levels in Newborns

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ABSTRACT

Objective: Most adipose tissue programming is realized in early life. Also, the postnatal three months, rather than the later phases of infancy, may be more relevant in the development of an adverse cardiometabolic risk profile. The adipokines phenotype, as a predictor of early-life weight gain, has been recently explored in cord blood. To determine whether in addition to leptin levels in cord samples, adiponectin, interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), resistin, plasminogen activator inhibitor-1 (PAI-1), and tumor necrosis factor alpha (TNF-α) levels improve weight gain prediction during the first three months of life.

Methods: Adiponectin, IL-6, MCP-1, leptin, resistin, PAI-1, and TNF-α were measured by multiplex immunoassay in a subsample of 86 healthy term newborns.

Results: Leptin levels significantly predicted weight gain at 3 months of follow-up (r2=0.09, p=0.006). In the multivariate analysis, including additional adipokines in the model, stepwise or all at once, did not increase the prediction of weight gain after the first three months of life.

Conclusion: Adding adiponectin, IL-6, MCP-1, resistin, PAI-1, and TNF-α to the prediction model of weight gain in healthy newborns did not prove to be useful. It is probable that their relative contribution to weight gain is not important. Only leptin was relevant as a predictor of weight gain at the 3-month endpoint.

Keywords: Leptin, adipokines, prediction, weight gain, newborn

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

The adipokines phenotype, as a predictor of early-life weight gain, has been recently explored in cord blood serum concentrations. Leptin levels significantly predicted weight gain at 3 months of follow-up.

WHAT THIS STUDY ADDS?

Adding adiponectin, interleukin-6, monocyte chemoattractant protein-1, resistin, plasminogen activator inhibitor-1, and tumor necrosis factor alpha to the prediction model of weight gain in healthy newborns was not useful.

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Introduction

Most of adipose tissue programming and therefore, adulthood obesity, has its origin in early stages of life; i.e. during intrauterine life and the breastfeeding period (1,2,3). Accelerated growth during the first 24 months of life has been associated with an adverse cardiometabolic profile and metabolic syndrome (4). Within these first two years of life, the initial three months may be more relevant in the development of an adverse cardiometabolic risk profile (5,6).

The adipokines phenotype as predictor of early-life weight gain, has been recently explored, both in breast milk and cord blood serum concentrations (7,8,9). Adiponectin and leptin are factors known to influence normal and abnormal fetal growth (10,11,12). Low adiponectin concentrations have been reported in adults and in infants who were born small for gestational age, linking growth restriction with a cardiometabolic profile later in life (13).

Other factors which have been identified in breast milk are normally secreted in adipocytes [interleukin-6 (IL-6); monocyte chemoattractant protein-1 (MCP-1), resistin, tumor necrosis factor-α (TNF-α)] (14). These additional factors in breast milk, have not yet been studied in cord blood as predictors of weight gain during the early postnatal period.

The aim of this study was to determine if, in addition to the leptin concentration in a cord sample, the cord blood levels of adiponectin, IL-6, MCP-1, resistin, plasminogen activator inhibitor-1 (PAI-1), and TNF-α improve the prediction of weight gain during the first three months of life in a cohort of healthy term newborn subjects.

Methods

A subsample of a previously reported cohort (3) of healthy normal weight at-term newborns is reported in this study. The original study population was composed of 99 infants born at the University Hospital of the Universidad Autónoma de Nuevo Leon in Monterrey, Mexico between January 2006 and December 2007. The subset is a convenience sample of those subjects from the original cohort whose blood cord serum of a sufficient quantity (25 µL) had been stored to run a multiplex immunoanalysis (MIA). Also, both baseline and at age 3 months (± one week) weight data were available for these subjects.

Weight was measured at birth, in duplicate, using a Torrey calibrated scale (Torrey, S.A. de C.V., Monterrey, Mexico) in the delivery room and during the 3-month follow-up visit at the outpatient clinic.

The cord blood samples were obtained from the umbilical cord vein immediately after birth and centrifuged at 3000 rpm. Serum was separated and stored at -20 °C in aliquots for later analysis. Samples were thawed only once at the time of the analysis.

The study was approved by the Ethics and Research Committee of the 'Dr. José Eleuterio González University Hospital of the Universidad Autónoma de Nuevo León. Parents gave written informed consent before data collection and blood sampling.

Adiponectin, IL-6, MCP-1, leptin, resistin, PAI-1, and TNF-α were measured by MIA using an ad doc human serum adipokine kit (96-well plate assay Cat# HADK2-61K-B, Millipore Corp, St. Charles, MO) on a Luminex TM 200 analyzer system (Luminex Corporation, Austin, TX). Sensitivity and intra-/inter-assay variation coefficients are shown in Table 1.

Statistical Analysis

Central tendency measures are expressed as mean ± standard deviation values unless otherwise specified. Those variables with a non-normal distribution were transformed to Ln for analysis. A stepwise linear regression analysis was performed to find variables in addition to leptin which may increase the accuracy of the weight-gain-at-month-3 prediction equation. A p-value ≤0.05 was considered statistically significant. All analyses were performed using IBM SPSS Statistics for Mac v.21.0 (Chicago, IL, USA).

Results

The study population included 86 term newborns (male n=41, 47.7%). The feeding type was available in 85 of the subjects (breastfeeding, n=43; formula-feeding n=42). Mean gestational age was 39.24±1.2 weeks. Mean birthweight was 3.31±0.45 kg. On average, the weight gain at age 3 months was 6.19±0.72 kg and 88.94% of the baseline weight (2.87±0.63 kg) (Table 2). No signs of morbidity were encountered in the study sample during the 3-month follow-up period.

Table 1. Sensitivity and intra-/inter-assay coefficients of variation

Cytokine	Sensitivity (pg/mL)	Intra-assay variation CV (%)	Inter-assay variation CV (%)
Adiponectin	21	4	10
IL-6	1.6	7.8	18
MCP-1	5.8	5	14
Leptin	85.4	7.9	15
Resistin	0.14	7.9	18
PAI-1	4.4	3	14
TNF-α	0.14	7.8	16

pg: picograms, CV: coefficient of variance, IL-6: interleukin-6, MCP-1: monocyte chemoattractant protein-1, PAI-1: plasminogen activator inhibitor-1, TNF-α: tumor necrosis factor α

Table 2. Neonatal characteristics of the study group (n=86)

Characteristic	Mean ± standard deviation
Gender	
Male, n (%)	41 (47.7%)
Female, n (%)	45 (52.3%)
Type of feeding	
Breastfeeding, n (%)	43 (50.6%)
Formula-feeding, n (%)	42 (49.4%)
Mean gestational age (weeks)	39.24±1.20
Mean birth weight (kg)	3.31±0.45
Mean birth length (cm)	49.68±1.97
Mean weight gain at 3 months (%)	88.94±25.08

Table 3. Adiponectin, interleukin-6, monocyte chemoattractant protein-1, leptin, resistin, plasminogen activator inhibitor-1, and tumor necrosis factor α concentrations in the umbilical cord blood of the subjects

Adipokine	n	Mean ± standard deviation
Adiponectin, µg/mL*	82	3.79±0.94
IL-6, pg/mL*	82	1.99±1.27
MCP-1, pg/mL*	83	5.92±0.53
Leptin, ng/mL*	85	3.53±0.91
Resistin, ng/mL*	81	4.10±1.40
PAI-1, ng/mL	83	53.37±16.30
TNF-α, pg/mL	84	52.29±4.63

*Expressed as Ln (natural logarithm) values; µg: microgram, pg: picograms, IL-6: interleukin-6, MCP-1: monocyte chemoattractant protein-1, PAI-1: plasminogen activator inhibitor-1, TNF-α: tumor necrosis factor α

Adiponectin, IL-6, MCP-1, leptin, and resistin were transformed to Ln for analysis. TNF-α and PAI-1 had a normal distribution and did not require transformation. Baseline Ln adiponectin was 3.79±0.94 µg/mL; Ln IL-6 was 1.99±1.27 pg/mL; Ln MCP-1 was 5.92±0.53 pg/mL; Ln leptin levels were 3.53±0.91 ng/mL; Ln resistin was 4.1±1.4 ng/mL; TNF-α was 52.29±4.63 pg/mL; and PAI-1 was 53.37±16.3 ng/ mL (Table 3).

Leptin levels weakly but significantly predicted weight gain at 3 months for the whole population (r2=0.09, p=0.006). In the multivariate analysis, including either a stepwise or an all at once approach, the additional adipokines available to the model did not increase the prediction of weight gain after the 3-month follow-up period. When analysing the population by feeding type during the 3-month period, no differences were found vs. the full cohort analysis.

Discussion

It has been demonstrated that rapid weight gain in both small-for-gestational age and appropriate-for-gestational age full-term infants during the first 3 months of life is associated with insulin resistance and hypertriglyceridemia in adult life (6). In a twins study, the relative influence of both environmental and intrinsic subject characteristics has been analysed, and both components were reported to have an association with cardiovascular risk factors in adult life (5). In a previous analysis, we reported the association of cord blood leptin levels and type of feeding with weight gain at 3 months of life (15). In recent years, the technology for cytokine measurement has evolved and became more accessible. Also, a lower sample volume is required to conduct multiple analyses simultaneously (16). We therefore foresaw an opportunity to explore whether adding other cytokines to the prediction model would increase its efficacy or not.

Other approaches to evaluate the prediction of weight gain in different time windows have been reported. Nakano et al (17) found that adiponectin levels in cord serum significantly predict body mass index (BMI) z-score gains from birth to 3 years of age in Japanese infants; however, when only the first 6 months were analysed, it was found that adiponectin levels do not predict postnatal BMI z-score gains, a finding that suggests that energy intake influenced by breast feeding and leptin levels may be stronger factors that affect changes in BMI z-scores than adiponectin levels during this period. Meanwhile, Mazaki-Tovi et al (13) reported that adiponectin concentrations negatively correlate with weight and BMI at one year of age, and that leptin concentrations positively correlate with weight and size at one year, suggesting the role of both adipokines in postnatal growth. Mantzoros et al (9) concluded that low leptin levels in cord blood are associated with pronounced weight gain in the first six months of life and with a higher BMI at 3 years of age, while adiponectin levels in cord blood are inversely associated with weight gain in the first 6 months of life and predict an increase in central adiposity at 3 years of age. These findings highlight the fact that the prenatal and early postnatal period are of developmental plasticity. It can be speculated that during such critical periods, long-term metabolic pathways that become relatively resistant to change are activated.

The association between adiponectin and weight at one month of age was not demonstrated by Inami et al (18), suggesting that adiponectin has an effect on fetal growth but no effect on early postnatal growth, at least in the first month of life.

It is important to point out the large diversity in the methodologies as well as in the study populations, and time periods of these studies could explain the inconsistency of the results.

In our study, the variance of weight gain explained by leptin was 9%, while the addition of adiponectin, IL-6, MCP-1, resistin,

PAI-1, and TNF-α to the model explained a variance of 11.7%, this latter finding remaining significant only by determining leptin as a predictor. Thus, the addition of these adipokines to the model in our study did not prove useful in predicting weight gain in three-month term newborns born with appropriate weight for gestational age. However, in subjects at the extremes of birth weight or in other time periods, these molecules may play a role in explaining weight gain in our study group, subjects that were essentially healthy, but it is unlikely that their relative contribution to weight gain is important.

Given that in our study population, both feeding types (namely breastfeeding and formula-feeding) are similarly represented, that both genders are equally distributed, and that the study population was intentionally selected as a healthy term-newborn population with normal weights for gestational age and that epidemiologic, nutritional and stress-related factors were not perceived to bias the study outcome, it is fair to state that among the cytokines studied, only leptin was relevant as a predictor of weight gain at the 3-month endpoint. Although this was a negative study with regard to the added value of other cytokines to the model, it is an important contribution to the understanding of weight change in the early developmental period.

Ethics

Ethics Committee Approval: The study was approved by the Ethics and Research Committee of the 'Dr. José Eleuterio González University Hospital of the Universidad Autónoma de Nuevo León, Informed Consent: Parents gave written informed consent before data collection and blood sampling.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Manuel E. de la O. Cavazos, Consuelo Treviño-Garza, Design: Consuelo Treviño-Garza, Isaías Rodríguez-Baderrama, Fernando F. Montes-Tapia, Data Collection or Processing: Consuelo Treviño-Garza, Laura Villarreal-Martínez, Jesús Z. Villarreal-Pérez, Analysis or Interpretation: Consuelo Treviño-Garza, Leonardo Mancillas-Adame, Cynthia M. Estrada-Zúñiga, Literature Research: Consuelo Treviño-Garza, Leonardo Mancillas-Adame, Cynthia M. Estrada-Zúñiga, Writing: Consuelo Treviño-Garza, Leonardo Mancillas-Adame, Cynthia M. Estrada-Zúñiga.

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Original Article

The Association between Serum 25-Hydroxy Vitamin D Level and Urine Cathelicidin in Children with a Urinary Tract Infection

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ABSTRACT

Objective: Cathelicidin is an important antimicrobial peptide in the urinary tract. Cathelicidin expression is strongly stimulated by 1,25-dihydroxy vitamin D in epithelial cells, macrophages/monocytes, and neutrophils. Vitamin D and cathelicidin status in children with urinary tract infection (UTI) caused by *Escherichia coli* is unknown. To establish the relationship between serum vitamin D and urine cathelicidin levels in children with a UTI caused by *Escherichia coli*.

Methods: Serum 25-hydroxy vitamin D and urine cathelicidin levels were measured in 36 patients with UTI (mean age 6.8±3.6 years, range: 0.25-12.6 years) and 38 controls (mean age 6.3±2.8 years, range: 0.42-13 years).

Results: There were no significant differences in urine cathelicidin levels between the study and control groups (p>0.05). Eight (22.2%) patients in the study group and 21 (58.3%) children in the control group were found to have sufficient vitamin D (≥20 ng/mL). Patients with sufficient vitamin D had higher urine cathelicidin levels than the controls with sufficient vitamin D (respectively 262.5±41.1 vs. 168±31.6 ng/mL, p=0.001). There were no significant differences between the patients and controls with insufficient vitamin D (p>0.05).

Conclusion: The children with vitamin D insufficiency may not be able to increase their urine cathelicidin level during UTI caused by *Escherichia coli*. There is a need of prospective studies in order to prove a beneficial effect of vitamin D supplementation for the restoration of cathelicidin stimulation and consequently for prevention of UTI recurrence.

Keywords: Urinary tract infection, *Escherichia coli*, children, vitamin D, cathelicidin

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

A vitamin D insufficiency can affect the defense system through multiple pathways and lead to infection. Cathelicidin plays many roles protecting the urinary tract from infection. Cathelicidin expression is strongly stimulated by 1,25-dihydroxy vitamin D in epithelial cells, macrophages/monocytes, and neutrophils.

WHAT THIS STUDY ADDS?

The children with vitamin D insufficiency may not be able to increase their urine cathelicidin level during urinary tract infection caused by *Escherichia coli*.

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Introduction

Urinary tract infection (UTI) is one of the most commonly acquired bacterial infections in childhood. It affects 10% of children and causes significant morbidity (1). *Escherichia coli* (*E. coli*) is the predominant pathogen in childhood UTI found in 90% of girls and in 80% of boys at the primary UTI. An important factor for the predominance of *E. coli* is their ability to attach to the urinary tract endothelium (2).

Vitamin D deficiency and insufficiency is a global issue (3). It is well known that vitamin D is involved in classical calcium homeostasis. More recent data from a variety of sources indicate that vitamin D has a broad spectrum of actions against autoimmune diseases and infections (4,5). It was shown that recurrent UTIs in premenopausal women are associated with vitamin D deficiency (6). Recently, it was reported that serum 25-hydroxy vitamin D level of <20 ng/mL was associated with UTI in children (7). Furthermore, the vitamin D receptor gene polymorphism was an important factor for UTI susceptibility in a study of children diagnosed with UTI and in this study, the most commonly isolated agent from urinary cultures was *E. coli* (8). Although it is not yet known the mechanism between the vitamin D deficiency and infection, a vitamin D insufficiency can affect the defense system through multiple pathways and lead to infection. Unfortunately, we did not evaluate all of the factors in the urinary tract defense system with which vitamin D is associated.

It has been speculated that urinary tract defense may be profoundly dependent on specific soluble epithelial cell-derived mediators and one of them are inducible bactericidal antimicrobial peptides, such as α- and β-defensins and cathelicidin (9,10). Among them, cathelicidin is involved in protecting the urinary tract from infection. Cathelicidin may stimulate production of chemokines and cytokines by several cell types and it also plays an important role in maintaining urinary tract integrity (11,12,13). Furthermore, cathelicidin expression is strongly stimulated by 1,25-dihydroxy vitamin D in epithelial cells, macrophages/monocytes, and neutrophils (11).

Vitamin D and cathelicidin status in children with UTI is unknown. Therefore, in this study, we analyzed serum vitamin D and urine cathelicidin levels in children with a UTI caused by to *E. coli*.

Methods

Patients

This study was approved by the local ethics committee and performed in accordance with the Declaration of Helsinki. Informed consent was obtained. Participants were recruited in the Pediatric Department from April to July 2014. The study group consisted of children with a UTI. The control group consisted of 38 healthy, age- and sex-matched children. Exclusion criteria were (i) prior UTI history; (ii) congenital anatomical anomalies such as meningomyelocele, cardiac abnormalities requiring surgery, skeletal dysplasia, renal hypo-dysplasia, cystic kidney disease, midline defects; (iii) chronic diseases and drug use such as to treat diabetes mellitus, epilepsy, bronchial asthma, chronic renal failure, hypertension; (iv) previous infections in the 6 weeks before the study; (v) obesity or malnutrition (vi) permanent urinary catheter, urinary tract stent or nephrostomy tube, urinary incontinence, neurogenic bladder; (vii) asymptomatic bacteriuria; (viii) kidney malformations and kidney stones; (ix) vesicoureteral reflux (VUR); and (x) chronic drug use or vitamin D supplementation. Diagnostic criteria for an upper UTI were fever, at least one UTI symptom (flank pain or costovertebral tenderness), pyuria, and presence of *E. coli* in the urine. Diagnostic criteria for a lower UTI were at least one UTI symptom (dysuria, urgency, suprapubic pain, irritability), pyuria, and presence of *E. coli* in the urine. We defined a pyuria as ≥5 white blood cells (WBCs) per high-power field on a spun urine. Urine samples were obtained by the midstream clean catch method for toilet-trained children, by urinary catheters for febrile infants and small children, and by bag for afebrile infants and small children. Presence of ≥50.000 cfu/mL of *E. coli* by catheterization or $\geq 10^5$ cfu/mL *E. coli* by mid flow urine were considered indicative of a positive urine culture (14). In a bag sample, if the urinalysis result was positive for pyuria in a symptomatic patient and there was a single organism cultured with a colony count >100,000, UTI was diagnosed (15).

All patients underwent renal and bladder ultrasonography within 48 hours of admission. A voiding cystourethrogram (VCUG) was considered to be indicated if renal and bladder ultrasonography revealed hydronephrosis or other findings that would suggest either high-grade VUR or obstructive uropathy. Other atypical or complex clinical or laboratory findings (poor urine flow, abdominal or bladder mass, raised creatinine level, septicemia, failure to respond to correct antibiotic treatment within 48 h, presence of VUR in first-degree family members, infection with non-*E. coli* organisms) were also accepted as indications for VCUG (16,17).

Study Design

This was a cross-sectional prospective study that examined the association between 25-hydroxy vitamin D and urine cathelicidin levels in children with UTI and in a healthy control group. Serum and urine samples were obtained before UTI treatment. Demographic variables (age and sex), disease type (lower or upper UTI), results of urinalysis, blood levels for C-reactive protein, white blood cell count, serum vitamin D, and urine cathelicidin levels were recorded. The serum samples were protected from light and stored at -80 °C prior to analysis. Serum 25-hydroxy vitamin D levels were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (DIAsource, Louvain-la Neuve, Belgium).

Vitamin D deficiency has been defined by the Institute of Medicine (IOM) as a measured serum 25-hydroxy vitamin D <12 ng/mL, vitamin D insufficiency when serum 25-hydroxy vitamin D is between 12 and 20 ng/mL, and vitamin D sufficiency is defined as a 25-hydroxy vitamin D level of 20 ng/mL (18).

Clean urine samples were taken midstream and in bags. Urine samples were centrifuged for 20 min at 2000-3000 rpm, and the supernatant was removed. The specimens were stored at -80 °C

prior to analysis. Urine cathelicidin levels were measured using a commercial ELISA kit (Eastbiopharm, Hangzhou, China). The kit uses a double-antibody sandwich ELISA to assay the human cathelicidin-1 level in the sample. Cathelicidin-1 was added to wells pre-coated with human cathelicidin-1 monoclonal antibody. After incubation, biotin-labeled cathelicidin-1 antibody was added and combined with streptavidin- horseradish peroxidase to form an immune complex. The color change, known to be positively correlated with the human cathelicidin-1 concentration in the sample, was measured spectrophotometrically at 450 nm. The intra-assay coefficient of variation (CV) was <10%, and the inter-assay CV was <12%. The assay range was 7-300 ng/mL, and sensitivity was 3.21 ng/mL.

Statistical Analysis

All statistical calculations were carried out using SPSS for Windows ver. 15.0 (SPSS, Inc., Chicago, IL, USA). Simple between-group comparisons were made using Student's t-test. The parameters are expressed as mean values and standard deviations. Spearman's rank test was used for the correlation analysis. A p-value <0.05 was considered statistically significant.

Results

The study group consisted of 36 children (6 males; mean age, 6.8±3.6 years; range, 0.25-12.6 years). Thirty-eight children (10 males; mean age, 6.3±2.8 years; range, 0.42-13 years) served as the control group. No significant differences in age or sex were observed between the study and control groups. Vitamin D levels differed significantly between the groups (p<0.05); however, no differences in urine cathelicidin levels were detected. The demographic and biochemical values of the study and control groups are presented in Table 1.

Eleven (30.5%) patients had an upper UTI (2 males; mean age, 4.7±2.5 years; range, 0.25-10 years) and 25 (69.4%) patients had a lower UTI (4 males; mean age, 7.4±3.2 years; range, 1.1-12 years). There were significant differences in age between patients with upper and those with lower UTIs (p=0.013). No significant differences were observed between patients with upper and lower UTI in gender, vitamin D and cathelicidin levels, or presence of pyuria. There was no correlation between the urine cathelicidin and pyuria level in the patient group (p=0.794, r=-0.045).

Eight (22.2%) patients in the study group and 21 (58.3%) children in the control group had sufficient vitamin D levels (≥20 ng/mL). Twelve (33.3%) patients in the study group and 15 (39.5%) children in the control group had vitamin D levels between 12-19 ng/mL. Sixteen (44.4%) patients in the study group and two (5%) children in the control group had vitamin D deficiency (<12 ng/mL). Patients with an adequate vitamin D level (n=8) had a higher mean urine cathelicidin level than did the controls with sufficient vitamin D (n=21) (p=0.001). There were no significant differences for urine cathelicidin level between the patient and control groups with insufficient vitamin D (p>0.05). The demographic and cathelicidin values of these patients are presented in Table 2. There was no correlation between the urine cathelicidin level and pyuria in the patient group with insufficient vitamin D nor in those with sufficient vitamin D (p>0.05).

Using chi-square test (vitamin D insufficient/sufficientxUTI/control), findings revealed there was a dependency relationship between vitamin D status and UTI (x2counted=7.139, x2expected=14.11, SD=1, p=0.008).

A positive correlation was found between vitamin D and urine cathelicidin levels in the study (n=36, p<0.0001, r=0.587, Figure 1a) and control groups (n=38, p=0.02, r=0.367, Figure 1b).

Cathelicidin levels were higher in the vitamin D sufficient (n=29) group as compared to the vitamin D insufficient (n=45) group. These levels were respectively 169.17±28 vs. 153.87±32 ng/mL, p=0.038 in these two groups. A positive correlation was found between vitamin D and urine cathelicidin levels in the vitamin D sufficient group (p=0.022, r=0.307). However, there was no correlation between vitamin D and urine cathelicidin levels in the vitamin D insufficient group (p=0.372).

Discussion

Effects of vitamin D beyond the skeletal system are becoming increasingly important. In this study, we examined the relationship between serum levels of vitamin D and urinary cathelicidin for the first time in children with a UTI. We found that urine cathelicidin level did not increase significantly during a UTI in children with vitamin D insufficiency.

Recently, it was reported that a serum 25-hydroxy vitamin D level of <20 ng/mL was associated with UTI in children (7). In our study, frequency of vitamin D insufficiency was significantly higher in children with a UTI than in those in the control group. In a study conducted on premenopausal women, it was found that uncomplicated UTI caused by *E. coli* lead to an increase in urinary cathelicidin levels during infection compared to postinfection levels (19). It was also shown that cathelicidin expression and secretion were increased during *E. coli* urinary tract colonization in children with cystitis or pyelonephritis (12). However, we found no differences in the cathelicidin levels between the study and control groups which may have been associated with the state of vitamin D insufficiency in this group of subjects. When the groups were divided according to their vitamin D levels, patients with sufficient vitamin D levels had higher cathelicidin levels than the controls who also were vitamin D sufficient. On the other hand, there were no significant differences in urine cathelicidin

Table 1. Demographic and biochemical characteristics of the study and control groups

	Study group (n=36)	Control group (n=38)	p-value
Sex (M/F)	6/30	10/28	>0.05
Age (years)	6.8±3.6	6.3±2.8	>0.05
Vitamin D (ng/mL)	16.5±6.3	23.7±11	0.03*
Urine cathelicidin (ng/mL)	181.9±59	164.5±27.8	>0.05

Data are presented as mean ± standard deviation values, M: male, F: female, *p<0.05

Table 2. Demographic characteristics and cathelicidin values of the groups according to vitamin D status				
		Study group (n=36)	**Controls (n=38)**	**p-value**
Vitamin D sufficient group*	Number of children	8	21	
	Age (years)	3.8±3	5.7±3.2	>0.05
	Gender (M/F)	2/6	6/15	>0.05
	Urine cathelicidin (ng/mL)	262.5±41.1	168±31.6	0.001*
Vitamin D insufficient group**	Number of children	28	17	
	Age (years)	7.3±2.9	7.1±2.1	>0.05
	Gender (M/F)	4/24	4/13	>0.05
	Urine cathelicidin (ng/mL)	155.8±37.4	156.2±21.2	>0.05
Data are presented as mean ± standard deviation values, M: male, F: female, *p<0.05 Vitamin D serum level >20 ng/mL; **Vitamin D serum level <20 ng/mL				

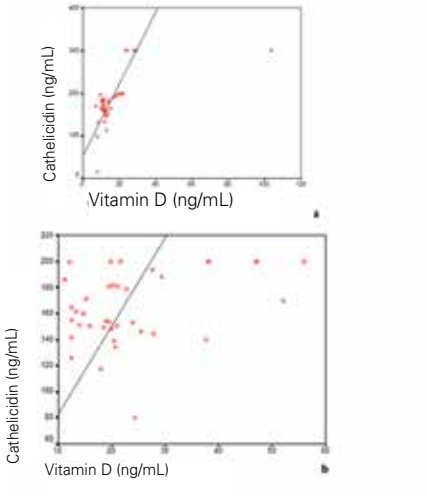

Figure 1. (a, b) Correlations between vitamin D and urine cathelicidin level. a) There was a positive correlation between vitamin D and urine cathelicidin levels in the study group (n=36, p<0.0001, r=0.587). b) There was a positive correlation between vitamin D and urine cathelicidin level in the control group (n=38, p=0.02, r=0.367)

level between patients with insufficient vitamin D and the controls with insufficient vitamin D. According to these findings, vitamin D insufficiency did not lead to an increase in the urine cathelicidin level during UTI in these children. These results suggest that sufficient vitamin D may be required to increase urine cathelicidin levels during a UTI.

Our findings revealed there is a dependency relationship between vitamin D status and UTI. Although this relationship is not exactly defined, the 25-hydroxy vitamin D is known to have an effect on the urothelium, with immunomodulatory capacity against *E. coli* infection (11,12,20). Recently, it was demonstrated that during pregnancy, increases in 25-hydroxy vitamin D and cathelicidin levels were observed as pregnancy advanced and, as gestation advanced, serum had an increased capacity to inhibit *E. coli* growth in urothelial cells (21). In a study of postmenopausal women, after vitamin D supplementation, increased cathelicidin expression in bladder biopsy samples with *E. coli* infection was observed compared with prior to supplementation (20). In this present study, we also established a positive correlation between vitamin D and cathelicidin in vitamin D-sufficient children. These results suggest that the effect of serum vitamin D on cathelicidin expression in the urinary tract depends on the level of vitamin D.

The major sources of cathelicidin in the urinary tract are circulating neutrophils, renal cells, and uroepithelial cells (12). A positive correlation has previously been observed between cathelicidin level and pyuria (12). However, we did not find a similar correlation in sufficient and insufficient vitamin D patients. In a study of cathelin-related antimicrobial peptide (CRAMP; an ortholog of the sole human cathelicidin) deficient mice, it was found that CRAMP-deficient hosts demonstrated less intense cytokine responses, diminished neutrophil infiltration, and accelerated uroepithelial recovery (22). Thus, it would seem that CRAMP may enhance *E. coli* infection in the bladder by promoting local inflammation. However, the authors pointed out that in total, their data indicate activities for cathelicidin during UTI that are independent of its direct antimicrobial activity. It appears that we need more studies for multiple biological activities during host-pathogen interactions.

It was recently reported that the frequency of vitamin D insufficiency in Turkish children and adolescents was 40% (23). The frequency of vitamin D insufficiency was similar in our control group.

Accumulated knowledge about cathelicidin and its relationship with urinary infection has been associated with *E. coli*. We therefore, in this study, preferred to include patients with UTI caused by *E. coli* to ensure homogeneity.

This study had some limitations. The sample size was low. This was a cross-sectional study; therefore, we do not know the previous urine cathelicidin status of the patients. A prospective study is needed to examine the beneficial effect of vitamin D supplementation to restore cathelicidin expression and prevent UTI recurrence.

The recommended vitamin D doses support bone health but also seem to be useful for fighting infection. Our results suggest that adequate vitamin D may benefit the urinary tract during a UTI by inducing cathelicidin expression.

In conclusion, our main finding was that the urine cathelicidin level was significantly upregulated in children with UTI and sufficient vitamin D status. In contrast, urine cathelicidin levels did not increase significantly during a UTI in children who had vitamin D insufficiency. Vitamin D deficiency and insufficiency is a global issue. Determining the vitamin D status of children with a UTI history and supplementing to restore proper vitamin D levels is a simple and cheap approach. The vitamin D-cathelicidin pathway is a human/primate-specific process; therefore, additional human studies should reveal the appropriate vitamin D level that is most beneficial during a UTI.

Ethics

Ethics Committee Approval: This study was approved by the local ethics committee and performed in accordance with the Declaration of Helsinki, Informed Consent: Informed consent was obtained.

Peer-review: Externally peer-reviewed.

Authorship Contributions

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Original Article

Endocrine Dysfunctions in Patients with Inherited Metabolic Diseases

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ABSTRACT

Objective: Inherited metabolic diseases (IMDs) can affect many organ systems, including the endocrine system. There are limited data regarding endocrine dysfunctions related to IMDs in adults, however, no data exist in pediatric patients with IMDs. The aim of this study was to investigate endocrine dysfunctions in patients with IMDs by assessing their demographic, clinical, and laboratory data.

Methods: Data were obtained retrospectively from the medical reports of patients with IMDs who were followed by the division of pediatric metabolism and nutrition between June 2011 and November 2013.

Results: In total, 260 patients [139 males (53%) and 121 females (47%)] with an IMD diagnosis were included in the study. The mean age of the patients was 5.94 (range; 0.08 to 49) years and 95.8% (249 of 260 patients) were in the pediatric age group. Growth status was evaluated in 258 patients and of them, 27 (10.5%) had growth failure, all cases of which were attributed to non-endocrine reasons. There was a significant correlation between growth failure and serum albumin levels below 3.5 g/dL (p=0.002). Only three of 260 (1.1%) patients had endocrine dysfunction. Of these, one with lecithin-cholesterol acyltransferase deficiency and another with Kearns-Sayre syndrome had diabetes, and one with glycerol kinase deficiency had glucocorticoid deficiency.

Conclusion: Endocrine dysfunction in patients with IMDs is relatively rare. For this reason, there is no need to conduct routine endocrine evaluations in most patients with IMDs unless a careful and detailed history and a physical examination point to an endocrine dysfunction.

Keywords: Inherited, metabolic diseases, endocrine dysfunction, children

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Inherited metabolic diseases (IMDs) can affect many organ systems, including the endocrine system.

WHAT THIS STUDY ADDS?

There is limited data regarding endocrine dysfunctions related to IMDs in adults, but no data exist in pediatric patients with IMDs.

Introduction

The clinical disorders that arise from a single gene defect and develop as a consequence of a blockage of the metabolic pathways are accepted as inherited metabolic diseases (IMDs).

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Metabolic disorders fall into three distinct groups depending on their occurrence mechanism-intoxication type, energy deficit type, and disorders affecting the degradation of complex molecules (1).

Patients with a metabolic disease may have serious endocrine problems. Many endocrine glands are affected; however, diabetes mellitus (DM), thyroid dysfunction, and gonadal deficiency are more frequent (2). Endocrine disorders, especially DM, also tend to be more common in patients with mitochondrial diseases (3). It is notable that many latent endocrine dysfunctions may be concomitant with Fabry disease and could be life threatening (4). As IMDs may be accompanied by serious endocrine dysfunctions, being aware of the condition and early diagnosis by simple tests are crucial to preventing possible complications (2).

In this study, we aimed to perform an endocrine assessment in patients diagnosed with IMDs.

Methods

Ethical approval from the local ethics committee was obtained before the study. The data were collected from the records of 260 patients with IMDs followed in our unit between June 2011 and November 2013. The ages of the patients ranged from 0.08 to 49 years.

Demographic findings (i.e., age, gender), results of anthropometric measurements (i.e., height, weight, head circumference), and physical examination outcomes were evaluated. Annual growth rate and bone age of the patients were calculated. Patients with a height standard deviation score below -2 were accepted as having growth retardation. In patients with growth retardation, growth hormone (GH) deficiency was assessed by growth velocity, bone age, serum insulin-like growth factor 1 (IGF-1) value, and GH stimulation tests when needed.

Serum glucose and hemoglobin A1c levels of the patients were evaluated for DM. Serum sodium (Na) and potassium (K) levels were measured to assess mineralocorticoid functions of the adrenal gland. Adrenocorticotropic hormone (ACTH) and cortisol levels were used for the evaluation of glucocorticoid functions of the adrenal cortex. Serum thyroid-stimulating hormone (TSH) and free thyroxine (fT_4) levels were assessed for the evaluation of thyroid function. Serum calcium (Ca), phosphorus (P), and alkaline phosphatase (ALP) levels were used to evaluate bone metabolism. In addition, serum albumin levels were measured to assess the nutrition status of the patients.

For statistical analysis, SPSS for Windows version 16.0 software was employed. Shapiro-Wilk test was used to determine whether data distribution was normal. Arithmetic means ± standard deviations (SD) were calculated. Difference between categorical variables (frequencies) was assessed by the chi-square test. For continuous variables, means of two groups were compared with student's t-test. Correlation between variables was calculated using Pearson's coefficient. For significance, a=0.05 ($p<0.05$) was used.

Results

Of 260 patients with IMD, 139 (53%) were males and 121 (47%) were females. The numbers of the patients included in each of the main and sub-groups of disease are shown in Table 1. No significant differences regarding gender within the main ($p=0.145$) group and sub-groups ($p=0.232$) of disease were found. A total of 249 (95.8%) patients were in the pediatric age group and 11 (4.22%) were in the adult age group.

Growth retardation was observed in 27 of 258 patients (10.5%). Eight patients (29.6%) were in the cellular intoxication group, 11 (40.7%) were in the energy deficit group, and 8 (29.6%) were in the complex molecule accumulation-induced main disease group (Table 1). None of the patients with growth retardation had an abnormal GH/IGF-1 axis.

The proportion of patients who were on dietary protein restriction for their primary diagnosis of IMD was 21.5%. There was no correlation between protein restriction and presence of growth retardation ($p=0.712$); however, a significant correlation ($p=0.002$) was found between a serum albumin level below 3.5 g/dL and growth retardation. In addition, growth retardation was observed in 6 of 14 patients (42.9%) with a serum albumin level below 3.5 g/dL.

Only 2 (0.07%) patients with IMD had DM. The primary diagnosis of one of these two patients was lecithin-cholesterol acyltransferase (LCAT) deficiency and that of the other patient was Kearns-Sayre syndrome (KSS). Glucocorticoid deficiency was observed only in one (0.4%) patient with glycerol kinase deficiency. None of the patients with IMD had pubertal or GH disorders nor disorders related to thyroid, mineralocorticoid hormones, or bone disorders.

Discussion

In previous studies, mainly conducted on adult patients, endocrine disorders of various grades depending on enzyme activity and exposure time were observed in patients with IMDs. To our knowledge, the present study is the first investigation that included mostly pediatric cases with IMDs.

In IMDs, a short stature usually results from multiple causes. These may include liver failure, renal failure, malnutrition, psychosocial causes or primary disease (2). There are a small number of hypopituitarism-induced growth retardation cases reported in the relevant literature, such as a few mitochondrial cytopathies (5) and iron-overload diseases (6,7,8). Growth retardation may occur in approximately 30%-60% of patients with mitochondrial cytopathy, cystinosis, and galactosemia (2). In our study, the overall proportion of growth retardation was 10.5%; however, this figure was 25% in patients with mitochondrial disease, and no growth retardation was observed in our seven patients with galactosemia (Table 1).

In this study, although no significant correlation between protein restriction and growth retardation was found, a serum albumin level below 3.5 g/dL was found to correlate with growth retardation. There is a consensus in the literature that an

Table 1. Numbers of patients in the main and sub-groups of inherited metabolic diseases and percentages of cases with growth retardation in the sub-groups of diseases

Diagnosis	Number of cases (percentage)	Cases with growth retardation n (%)/N
1) Cellular Intoxication Type	**121 (47)**	**8 (6.6*)/120**
- Disorder of phenylalanine metabolism	77 (18)	3 (3.9)/76
- Disorder of amino acid metabolism	17 (6)	2 (11.7)/17
- Organic acidemias	11 (4)	2 (18.1)/11
- Urea cycle disorder	9 (3)	1 (11.1)/9
- Galactosemia	7 (3)	0 (0)/7
2) Energy Deficit Type	**76 (29)**	**11 (14.6**)/75**
- The cases with mitochondrial disease	29 (11)	7 (25.0)/28
- Biotinidase deficiency	25 (10)	0 (0)/25
- Glycogen storage disease	14 (5)	4 (28.5)/14
- Gluconeogenesis disorder	8 (3)	0 (0)/8
3) Complex Molecule Accumulation Type	63 (24)	8 (12.6***)/63
- Disorder of lipid metabolism	46 (30)	3 (6.5)/46
- Sphingolipidoses and peroxisomal disease	9 (4)	3 (33.3)/9
- Mucopolysaccharidosis and oligosaccharidoses	8 (3)	2 (25.0)/8
Total	**260 (100)**	**27 (10.5)/258**

For * and ** and *** **p<0.05**; for * and ** **p<0.05**; for * and *** **p<0.05**; for ** and *** p=0.07.

insufficient protein intake results in growth retardation (9). Hence, the main cause of growth retardation in cases with IMDs seems to be inadequate energy intake or strict protein restriction. To our knowledge, GH/IGF-1 axis in IMDs has not been evaluated so far. In our 27 cases with growth retardation, no abnormality was found concerning the GH/IGF-1 axis. An explanation for this finding might be that the growth retardation occurred independent of the GH/IGF-1 axis in our cases with IMDs. However, further studies should be conducted to find out the exact etiology of growth retardation in this group of the patients.

Many IMDs are accompanied by DM which usually occurs due to insulinopenia resulting from impaired pancreatic β-cell function (2). Among intoxication-type IMDs, DM develops due to iron overload in hemochromatosis (10) and in aceruloplasminemia (11), which may arise from ketoacidosis-induced pancreatitis in organic aciduria (12,13). In mitochondrial diseases (14,15) and glycogen storage diseases, DM is due to impaired β cell function arising from non-production of ATP (16). In addition, hypertriglyceridemia-induced pancreatitis may also contribute to the development of DM in glycogen storage diseases (17). In only 2 of 260 (0.07%) patients with IMDs, DM was diagnosed. One of these patients was diagnosed with LCAT deficiency of the lipid metabolism disorders, and another patient was diagnosed with KSS of the mitochondrial diseases. DM was previously described in both LCAT deficiency and KSS (2,18).

In adrenoleukodystrophy and in defects of energy metabolism of the IMDs, glucocorticoid deficiency may be observed (2). Among IMDs, X-linked adrenoleukodystrophy (X-ALD) is the disease that gives rise to primary adrenal insufficiency most frequently (19,20). Adrenal insufficiency occurs after the age of 3 years, which is likely to be the first indication of the disease. In Fabry disease, subclinical adrenal insufficiency may occur. Faggiano et al (4) found partial adrenal insufficiency in one of 18 patients with Fabry disease by using a corticotropin stimulation test. Mitochondrial disease-related adrenal insufficiency is rare in childhood, however, this may be the first symptom (21) associated with the poor prognosis (22,23,24). Glucocorticoid deficiency was detected in only 1 of 248 (0.4%) patients whose serum ACTH and cortisol levels were measured, and the diagnosis was glycerol kinase deficiency.

In conclusion, endocrine disorders of various grades depending on enzyme activity and exposure time can be observed in patients with IMDs. To our knowledge, this is the first study investigating endocrine functions in a group of patients with IMDs who were mostly of pediatric ages. In our study, an endocrine disorder was found only in 1.1% (3 of 260). GH/IGF-1 axis does not seem to be attributable to the growth retardation which was observed in 10.5% of the patients with IMDs. Serum albumin levels may be used in the follow-up of the patients to prevent growth retardation. We suggest that in pediatric patients with a diagnosis of IMD, routine endocrine tests are not necessary. Instead, both the patients and the IMD types should be evaluated individually, and endocrine tests should be done if required after obtaining a detailed history and performing physical examination including anthropometric and pubertal evaluation for the endocrine assessment.

Ethics

Ethics Committee Approval: This study was conducted based on approval from the Ethical Committee of Medical

Faculty of Uludağ University numbered 2013-18/32, as well as in accordance with the Declaration of Helsinki, Informed Consent: It was taken.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Şahin Erdöl, Halil Sağlam, Design: Şahin Erdöl, Halil Sağlam, Data Collection or Processing: Şahin Erdöl, Halil Sağlam, Analysis or Interpretation: Şahin Erdöl, Halil Sağlam, Literature Search: Şahin Erdöl, Halil Sağlam, Writing: Şahin Erdöl, Halil Sağlam.

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Original Article

The Role of Active Video-Accompanied Exercises in Improvement of the Obese State in Children: A Prospective Study from Turkey

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ABSTRACT

Objective: The aim of this study was to determine the effects of active video games and music-accompanied aerobic and callisthenic exercises on body mass index (BMI), body fat ratio, physical performance tests, psychosocial status, and self-respect in overweight and obese adolescents.

Methods: Fifty (21 males and 29 females) slightly overweight and obese participants with no chronic disorder and of an average age of 12.16±0.99 years were included in the study. The percentile values for BMI, triceps skinfold thickness, waist circumference measurements, and physical performance tests were evaluated. The effects of obesity on psychological wellness were evaluated using the depression scale for children (DSC) and the Piers-Harris Children's Self-Concept Scale for self-esteem. Following these evaluations, the participants were subjected to an exercise program in five groups of 10 people, 3 days a week for a duration of 8 weeks. Each exercise session lasted 45 minutes. Participants were re-evaluated at the end of the exercise program. The data collected both before and after the exercise program were analyzed using the SPSS 18.0 program.

Results: According to BMI reference values, 28% of the 50 participants (n=14; 6 males and 8 females) were assessed to be overweight and 72% to be obese (n=36; 15 males and 21 females). Following the exercise program, 14% of the participants (n=7; 3 males and 4 females) were assessed as normal, 46% (n=23; 14 males and 9 female) as slightly overweight, and 40% (n=20; 4 male and 16 female) as obese. It was determined that the decrease in BMI values ($p<0.05$) was higher in male participants than in female participants and that the frequency of obesity was higher in the females. A statistically significant decrease in BMI values was found after the exercise program ($p<0.01$). Following the exercise program, statistically significant differences have also been observed in the self-esteem ($p<0.01$), psychological wellness ($p=0.025$), triceps skinfold thickness, as well as in waist circumference and BMI values of the participants compared to the pre-exercise phase ($p<0.01$).

Conclusion: An exercise program applied with active video games was found to have positive effects on the obese state as well as on the psychosocial status and self-esteem of obese individuals, indicating that exercise and physical activity have an important role in improvement of the obese state in childhood as well as having positive contributions to self-esteem and psychological wellness state.

Keywords: Child obesity, active video game, exercise, self-esteem, depression

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Children at risk of overweight have been shown to benefit from increased physical activity administered as a structured program. However, children can get bored of exercise quickly so it is essential to make those exercises interesting.

WHAT THIS STUDY ADDS?

After applying an exercise program which was accompanied by active video games, we found significant difference in self-esteem, psychological wellness, performance tests, and body mass index compared to the pre-exercise phase.

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Introduction

Obesity is a result of an imbalance between nutrient intake and energy expenditure (1). Due to the influence of technology and ever changing social structure, people now spend increasing amounts of their time in front of the television or computer. The risk of obesity increases with a reduction in physical activity. Negative changes in dietary habits combined with a decrease in energy expenditure are the leading causes of this major public health problem. Obesity adversely affects quality of life because of its negative physiological, psychological, systematic, and social effects (2).

Obesity is a condition which leads to various health problems. Its frequency is increasing rapidly amongst adults, children, and adolescents all over the world (3,4). It has been reported that in developed countries, as many as 33% of adults and 20-27% children and adolescents are obese (5). According to the 2010 Turkish Nutrition and Health Research pre-work report, the frequency of obesity in the 15 years and older population is 20.5% among men, 41% among women, and 30.3% in total. The same report states that the frequency of obesity in Turkish children between the ages of 0 and 5 years is 8.5% (10.1% in boys and 6.8% in girls), and the frequency between the ages of 6 and 18 years is 8.2% (9.1% in boys and 7.3% in girls) (6). According to the Turkish Health Research issued by the Turkish Statistical Institute in 2014, the following body mass index (BMI) findings have been reported in the population 15 years and older: 33.7% overweight, 42.2% average weight, and 4.2% underweight. By gender, 24.5% women are obese and 29.3% are overweight, while 15.3% men are obese and 38.3% are overweight (7).

While determination of obesity among adults is calculated by BMI, this may not be appropriate for very young children. Weight and height values for age and gender are also evaluated in assessment of obesity in children. It is also important that local standard curves, if available, are used in this assessment (8).

Although many studies have been conducted to clarify the etiopathogenesis of obesity, all of them have pointed to a multiple etiology (9). Obesity causes various health conditions and chronic illnesses such as cardiovascular disease, hypertension, and diabetes in both adults and children (10,11). Studies have revealed that one third of those who were obese during their childhood and 80% of those who were obese in adolescence were obese as adults. It has also been reported that 30% of obesity cases among adults have a history of childhood obesity (12). Since obesity increases the risk of developing chronic diseases, mortality and morbidity, it is a significant risk factor for children (13).

Furthermore, being overweight and obese affects self-confidence, general appearance, and social activity. A study revealed that depression, lack of self-confidence, and behavior problems were more frequent among clinically obese adolescents (14). It has also been reported that obesity has adverse effects on depression and self-confidence among children (15).

Daily energy need varies with respect to age, gender, occupation, genetic, and physiological characteristics. In order to maintain a healthy weight, the amount of energy that is ingested needs to be balanced to the amount that is spent. Healthy nutrition and adequate physical activity have primary roles in maintaining this balance and in increasing quality of life. Regular physical activity is fundamental for children and adolescents to grow up to be a healthy adult. It is also helpful in avoiding acquisition of bad habits, in being social, and in protection from various chronic diseases (16).

Pharmacotherapy options for the treatment of pediatric obesity are very limited. Therefore, a comprehensive management program that includes exercise and behavior modification is crucial (17). It is essential to make exercise enjoyable and interesting for children because they can get bored of exercise quickly (18). In this study, we hypothesize that active video games and music-accompanied aerobic and calisthenic exercises will contribute positively to children's BMI percentile and psychosocial status.

Methods

This study was implemented within the scope of Supporting Scientists Program of the Scientific and Technological Research Council of Turkey (TÜBİTAK). After obtaining parental consent, primary school students were screened for suitability to participate in our study. Our target number was 50 overweight and obese participants. Evidence of chronic disease was an exclusion criterion.

During the evaluation process, the height and weight of the participants were measured using sensitive scales with sensitivities within 1 cm and 100 grams. BMI values were calculated as weight in kilograms divided by height in meters squared; children with BMI over the 85th percentile were included in the study. The reference percentile values for Turkish children were used in the evaluation of the data (13). Triceps skinfold thickness (in millimeters) measured by means of a caliper (Harpenden Anthropometry) and waist circumference (in centimeters) measured by means of a tape measure were used to determine body fat mass (19). Questionnaires were completed to determine age, gender, obesity history, possible chronic disease history, dietary habits, and problems that they might encounter during their daily life activities using face to face interviews. Physical performance tests were implemented to measure how many seconds it takes them to ascend and descend 20 stairs, the number of squats they can perform in 120 seconds, the amount of time they take to run 50 meters (in seconds), and the number of times they can jump over a rope in 30 seconds. The effects of obesity on psychological wellness were measured using the Depression Scale for Children (DSC)

and the effects of obesity on self-esteem were measured using the Piers–Harris Scale (20,21).

Body Mass Index

In children, BMI may vary with age, rate of growth, and pubertal stage (22,23). At present, gender-specific BMI-for-age percentile charts for the pediatric age group are available in many countries. According to these charts, children and adolescents with a BMI over the 85th but less than the 95th percentile for age and gender are considered as overweight and those with a BMI greater than the 95th percentile as obese (17). The International Obesity Task Force has published an international standard growth chart that enables comparison of prevalence globally (8,17). Nevertheless, many countries prefer to use their own country-specific growth chart because of national features (17). We used reference percentile values for Turkish children (13).

Waist Circumference

The waist circumference was measured via a tape measure at the umbilicus level (19).

Triceps Skinfold Thickness

The skinfold thickness can be measured at 10 different points on the body and they are considered as an accurate indication of body fat mass. The subscapular or the left triceps are suggested as the measurement sites in most reports. In this study, the triceps measurement was used and implemented at the exact middle point between acromion and olecranon by using a skinfold caliper, with the arm of the subject hanging vertically at the side (19).

Depression Scale for Children

DSC was developed by Kovacs (20) in 1992 to measure level of depression in children and adolescents between the ages of 6 and 17 years. This scale consists of 27 articles and evaluates the previous 2 weeks of the child's life. Each item is scored as 0, 1, or 2 with increasing numbers indicating increased level of depression. The cut-off score is 19 and individuals with scores above this score are accepted as depressed. The adaptation to Turkish validation and credibility were completed by Öy (24) in 1990.

Piers-Harris Scale

This scale was developed by Piers and Harris in 1964 to measure the self-concept of children between the ages of 9 and 16 years (21). Self-concept refers to the knowledge and perceptions that individuals have of themselves and their behavior. This scale measures the self-concept of children, the development of this concept, and its dimensions and relations with the environment. The answers that the child gives are scored and the child's self-conception is determined. The adaptation to Turkish validation and credibility was completed by Öner (25) in 1994.

The scale consists of 80 descriptive statements. The answers to those statements are either "Yes" or "No". The overall score ranges from 0 to 80. The individual's self-concept is at a more affirmative level as the scores increase. This scale can be completed within 15 to 20 minutes by children with normal development. The scale aims to determine the individual behavior of children and adolescents towards themselves, to study the correlation between these behaviors, and also to evaluate what they feel about themselves.

Exercise Program

The subjects were divided into five groups of 10 participants each and subjected to an 8-week exercise program for three days per week. The exercise program was as follows:

1) Warm-up exercises: Breathing exercises and slow-paced walking for 10 minutes.

2) Exercise program: Calisthenic and aerobic exercises aimed to target all muscle groups using visual biofeedback with music and active video games for 25 minutes. Three video games that last approximately 6 minutes each were used. Videos were displayed via projection devices and each of the dance moves for the music was demonstrated in the video by an instructor. Between the videos, breathing exercises were implemented combined with slow-paced walking with marked times.

3) Cool-down exercise: Mild stretching, slow-paced walking, and breathing exercises for 10 minutes. The program was completed with a relaxation posture.

The video games were transferred to CDs and distributed to the participants at the end of the 8-week training period in order to maintain the continuity of the exercises and enable them to acquire regular exercising habits.

The data acquired before and after the exercise program were evaluated using the SPSS 18.0 program (SPSS Inc., Chicago, IL, USA). The relationship between categorical variables was evaluated using the chi-square test, the relationship between gender and the continuous variables was evaluated using the Mann-Whitney U test, and the comparison of the data before and after the exercise program was evaluated using the Wilcoxon signed-rank test. A p-value <0.05 was considered statistically significant.

Results

We screened 80 adolescents for participation in the study and 50 participants met all the eligibility requirements and were included in the study (Table 1).

The dietary habits, daily meal times, and family obesity history of the participants were examined. It was determined that 56% of the study subjects were receiving a balanced diet and the remaining 44% did not. Number of meals per day was only 2 in 10% of the participants, 64% had 3 meals per day, 24% ate 4 meals per day, and 2% ate 5 or more times per

day. Examination of family history revealed that 42% of the participants had a first-degree relative with obesity.

We determined that 14 (28%) of the participants were overweight and 36 (72%) were obese before the exercise program. After the exercise program, the BMI values of the subjects had decreased significantly (p<0.05) (Table 2).

Table 1. The demographic characteristics of the participants (n=50)

			Age	Age
	n	%	X ± SD (years)	Range (years)
Female	29	58	12.66±0.96	11-14
Male	21	42	12.38±1.02	11-14
Total	50	100	12.16±0.99	11-14

X: mean, SD: standard deviation, n: number of subjects

Triceps skinfold thickness measurement values as well as waist circumference measurements also showed a decrease after the exercise program (p<0.05) (Table 3).

The 50 meter run time, the number of squats in 120 seconds (s), the time to ascend and descend 20 stairs, and the number of times jumping over a rope in 30 seconds had all improved after the exercise program (p<0.05) (Table 4).

Before the exercise program, 18 participants exhibited depressive findings, whereas after the exercise program, the number had decreased to 13. We determined that children demonstrated affirmative development in their psychological wellness after the exercise program (p<0.05) (Table 5).

A significant difference was recorded in the Piers-Harris Self-Respect sub-components of behavior, intelligence, school status, popularity, happiness, physical appearance, and anxiety levels after the exercise program (p<0.05) (Table 6).

Table 2. Classification of the participants based on body mass index before and after the exercise program

	Before the exercise program						After the exercise program					
	Male		Female		Total		Male		Female		Total	
BMI	n	(%)	n	(%)	**n**	**(%)**	n	(%)	n	(%)	**n**	**(%)**
Normal	0	0	0	0	**0**	**0**	3	(6)	4	(8)	**7**	**(14)**
Overweight	6	(12)	8	(16)	**14**	**(28)**	14	(28)	9	(18)	**23**	**(46)**
Obese	15	(30)	21	42	**36**	**(72)**	4	(8)	16	(32)	**20**	**(40)**
Total	21	(42)	29	(58)	**50**	**(100)**	21	(42)	29	(58)	**50**	**(100)**
p 0.000*												

*p<0.05, BMI: body mass index, n: Frequency

Table 3. Triceps fat mass and waist circumference measurements after the exercise program

	Before exercise program		After exercise program		
	X ± SD	Minimum-Maximum	X ± SD	Minimum-Maximum	p
Triceps skinfold thickness (mm)	19.74±4.82	13-32 mm	19.06±4.65	12-32 mm	**0.000***
Waist circumference (cm)	85.78±8.33	73-115 cm	83.66±8.30	71-115 cm	**0.000***

*p<0.05, X: mean, SD: standard deviation, mm: millimeters, cm: centimeters

Table 4. Physical performance tests before and after the exercise program

Before exercise program			After exercise program		
Physical performance tests	**X ± SD**	**Minimum-Maximum**	**X ± SD**	**Minimum-Maximum**	**p**
Run time for 50 meters (seconds)	15.24±1.48	12-18 s	14.48±1.46	11.50-18 s	**0.000***
Number of squats in 120 seconds (n)	69.32±11.13	40-90	74.52±10.86	42-92	**0.000***
Time up and down 20 stairs (seconds)	13.04±1.50	10-17 s	12.24± 1.55	9.80-16 s	**0.000***
Number of jumps over a rope in 30 seconds (n)	32.28±8.15	13-55	37.42±8.97	15-60	**0.000***

*p<0.05, X: mean, SD: standard deviation, n: frequency

Table 5. Depression status before and after the exercise program

Depression status		Before exercise program			After exercise program			p
		Gender		Total	Gender		Total	
		Male	Female		Male	Female		
Not depressive	n	13	19	32	14	23	37	0.025*
	%	61.9	65.5	64.0	66.7	79.3	74.0	
Depressive	n	8	10	18	7	6	13	
	%	38.1	34.5	36.0	33.3	20.7	26.0	
Total	n	21	29	50	21	29	50	
	%	42	58	100	42	58	100	
Median (minimum-maximum)		17 (9-29)	15 (8-28)		14 (5-25)	11 (4-22)		

***p<0.05**, n: frequency

Table 6. Piers-Harris Self-Respect Scale scores before and after the exercise program

Piers-Harris scale	Before exercise program X ± SD	After exercise program X ± SD	p
Intelligence and education	5.76±1.22	6.44±0.97	0.000*
Behavior	12.06±2.07	12.94±2.09	0.000*
Physical appearance	7.54±1.77	8.52±1.51	0.000*
Anxiety	9.32±2.17	10.06±1.77	0.000*
Popularity	8.34±1.55	9.02±1.62	0.000*
Happiness	9.28±2.09	10.20±1.96	0.000*
Total score	54.16±8.66	59.20±7.96	0.000*

***p<0.05**, X: mean, SD: standard deviation

In the evaluation form that we drew up to evaluate their dietary habits, the participants were asked whether they had a balanced nutrition, meaning eating natural homemade foods at regular times, and how many meals they had daily. Twenty-eight out of 50 participants (56%) answered "yes" when asked whether they had a balanced nutrition.

Upon evaluating the answers to these questions gender-wise, no statistical differences were recorded between the answers by the male and female participants.

Discussion

Improved diet, increased exercise, and change in eating habits as well as involving the family in the treatment process are essential to prevent the continuation of obesity. The addition of behavior treatment to the exercise and diet treatment already used to correct childhood/adolescent weight problems has proven to produce better results (3).

Since obese people are inclined to move less, exercise should not be neglected during their treatment. The purpose of exercise for obese people is not only to lose weight but also help them acquire a behavioral change for a healthier life style (26). For improvement of the obese state, it is essential that all adolescents exercise daily and accept it as part of their lives. Through exercise, the loss of fat increases and lean tissue mass is preserved (5).

By means of an 8-week exercise program, a highly significant decrease in triceps skinfold thickness and waist circumference measurements were observed in the participants of this study. These results may have long-term implications for our participants. Lack of physical activity leads uninformed adolescent and obese adults into a sedentary life which will increase the risk of cardiovascular, pulmonary, and metabolic diseases. Furthermore, obesity itself can cause chronic complications such as hypertension, stroke, cardiac diseases, thrombogenesis, pulmonary diseases, endometrial and colon cancers, sleep apnea, dyslipidemia, gallbladder stone, type 2 diabetes, gout and pain, or dysfunction in the locomotor system. These complications lead to a decrease in the life expectancy of the obese individual (15). Early intervention, such as that made in our study, may improve the quality of life of these individuals immediately, as shown by the changes in their self-esteem and depression scores.

In our study, 36% of the participants were found to be depressed. Their self-esteem was also observed to be low. These results agree with a study by Pınar (27) that examined the level of depression and self-esteem in 87 obese women. The author reported that 42.5% of the obese participants were depressed and 58.6% of them had low self-esteem. In addition, in a study by Kartal (28), it was demonstrated that obese individuals had a lower level of self-esteem. Following the exercise program, our participants demonstrated a positive development in their psychological

wellness (p=0.025). The number of our participants with depressive symptoms decreased from 36% to 26% after the exercise program. Moreover, compared to the pre-exercise program, a statistically significant improvement was recorded in the total Piers-Harris Self-Esteem points and their sub-components such as behavior, intelligence, education, popularity, happiness, physical appearance, and anxiety (p=0.000).

The effects of physical activity on obesity vary with age. For morbid obesity which may be accompanied by diabetes, cardiac disease, hypercholesterolemia, and arthritic diseases, low intensity exercises are suggested (25% VO_2 max). The exercises help burn esterified fatty acids. For obese people, exercises that do not involve a strain on body weight such as swimming, cycling, and mat exercises (exercises on the mattress) should be chosen. Ergometric exercises result in 10-12% fat loss. All the training programs should be rhythmic exercises that do not push the respiratory and circulatory systems too far. For other obese individuals, mildly intense exercises are advised (65% VO_2 max). These exercises stimulate the breakdown of intramuscular triglycerides. In addition, walking, dancing, treadmill use, water sports, gardening, and house chores are recommended (29).

In a randomized, controlled study which Maddison et al (30) carried out in 322 children (aged 10-14 years) in New Zealand, they applied 60 minutes of mildly intense physical activity during which the study group was exposed to active video games and the control group was exposed to non-active video games. They determined that the active video games had positive effects on the body composition and there was a significant increase in vital capacity.

In another study involving sedentary adolescents in New York, a comparison was made between a group that was exposed to a 30-minute active video game and another group that was given a 30 minute treadmill walk. It was observed that the video game group had a significant increase in their heart rate and pulse in comparison to the treadmill group, showing that active video games can be an alternative to aerobic exercises when it comes to physical activity (31).

Barbeau et al (32) demonstrated that a total of 80-minute activity program that consisted of 35 minutes aerobics, 20 minutes stretching, and 25 minutes skills development and which was offered every day during the school year, had significant effects on BMI and led to a decrease in body fat.

In this present study, an exercise program consisting of 10 minutes warm-up, 25 minutes aerobics exercise, and 10 minutes cool-down, which totaled 45 minutes and was accompanied by active video, was applied to participants for three days each week for 8 weeks. The exercise program resulted in significant decreases in the BMI percentile values of participants (p=0.022). Furthermore, we found that male participants had greater changes than female participants (Table 5). Generally in these age groups, boys are more interested in video or computer games than girls and this might be the reason for this result. In addition, physical performance tests revealed that there were significant improvements in the running time, stair climbing, number of squats and rope jumps compared to the pre-program findings (p=0.00).

In conclusion, the results of this study showed that the exercise program accompanied by active video that we applied had positive effects on the obese state as well as on the psychosocial status and self-esteem of our participants. In this study, exercises were made enjoyable for the participants, which caused them to become more active and willing. This finding was noted especially in the boys. We know that physical activity has a major role in preventing childhood obesity and can lead to a healthy and active lifestyle. Our study showed that active video game participation in exercise is effective for the improvement of the obese state and possibly for prevention of obesity in children.

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Ethics

Ethics Committee Approval: The study protocol was approved by the Ethics Committee of Mustafa Kemal University Faculty of Medicine at 13.01.2014, Informed Consent: Informed consent was obtained from all parents.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Fatma Duman, Mehmet Hanifi Kokaçya, Esra Doğru, Nihan Katayıfcı, Özden Canbay, Fatma Aman, Design: Fatma Duman, Esra Doğru, Nihan Katayıfcı, Özden Canbay, Data Collection or Processing: Nihan Katayıfcı, Özden Canbay, Fatma Aman, Analysis or Interpretation: Fatma Duman, Esra Doğru, Literature Search: Mehmet Hanifi Kokaçya, Esra Doğru, Nihan Katayıfcı, Writing: Fatma Duman, Mehmet Hanifi Kokaçya, Esra Doğru,Nihan Katayıfcı, Özden Canbay, Fatma Aman.

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Case Report

Identification and Functional Characterization of a Calcium-Sensing Receptor Mutation in an Infant with Familial Hypocalciuric Hypercalcemia

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ABSTRACT

Familial hypocalciuric hypercalcemia (FHH) is an autosomal dominant disorder, associated with inactivating mutations of the *calcium-sensing receptor* (*CaSR*). To evaluate the functional significance of a *CaSR* mutation, identified in a young infant who presented with hypercalcemia and hypocalciuria. The *CaSR* gene coding sequences were analyzed by polymerase chain reaction amplification and direct sequencing analysis. The mutation identified was introduced by site-directed mutagenesis into a wild-type (WT) CaSR plasmid, and human embryonic kidney 293 T cells were transfected with either the WT or mutant CaSR. The function of the mutated CaSR protein was analyzed by evaluating the free intracellular calcium [$(Ca^{2+})_i$] response after challenge with extracellular calcium (Ca^{2+}). We identified a heterozygous mutation c.772_773delGTinsA in exon 4 resulting in the substitution of amino acid valine (Val) with amino acid arginine (Arg) and the premature pause of the translation 46 amino acids later (Val258ArgfsTer47). Functional assay showed that cells transfected with the mutant CaSR had a significantly poorer response to extracellular Ca^{2+} stimulation compared with the WT. We have shown that the c.772_773delGTinsA mutation causes a significant alteration of CaSR function leading to features of FHH in an affected young infant since the first months of life.

Keywords: Familial hypocalciuric hypercalcemia, calcium-sensing receptor, calcium, hyperparathyroidism

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Familial hypocalciuric hypercalcemia (FHH) is associated with inactivating mutations of *calcium-sensing receptor* (*CaSR*) gene. FHH, in most of the cases, is a benign condition featured by asymptomatic hypercalcemia, however, diagnosis is essential in order to avoid unnecessary parathyroidectomy.

WHAT THIS STUDY ADDS?

We describe the identification of c.772_773delGTinsA mutation of CaSR and the impact on the clinical phenotype in a young infant harboring the mutation. Our functional analysis shows that c.772_773delGTinsA mutation is associated with significant impairment of *CaSR* function leading subsequently to the phenotype of FHH.

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Introduction

The calcium-sensing receptor (CaSR) is a G protein-coupled cell surface receptor expressed abundantly in parathyroid chief cells (1) and renal tubular cells (2). CaSR holds a principal role in the maintenance of serum calcium (Ca^{2+}) levels within a narrow range. Ca^{2+} is a mineral that plays a pivotal role in bone mineralization as well as in other physiological cell functions like intracellular signal transduction, hormone secretion, neurotransmitter release, and muscle cell contraction. The receptor recognizes any fluctuations in extracellular Ca^{2+} concentrations and finally modulates the parathyroid hormone (PTH) synthesis/secretion and Ca^{2+} renal reabsorption (3).

The human *CaSR* gene located in chromosome 3q13.3-21 contains 7 exons and encodes for a 1078 amino acid sequence (NM_000388). Exon 1 is untranslated, while exons 2-6 encode for a large extracellular N-terminal domain (ECD) and exon 7 for the seven transmembrane domains (TMDs) and the intracellular carboxy-terminal domain (ICD) (4). Loss-of-function mutations of the *CaSR* gene present in homozygotes with neonatal severe hyperparathyroidism (NSHPT), while heterozygotes develop familial hypocalciuric hypercalcemia (FHH) (5).

FHH is a rare autosomal dominant disorder, characterized by lifelong, usually asymptomatic hypercalcemia. The altered function of the receptor in this disorder decreases its sensitivity to extracellular Ca^{2+}, shifting the set point of Ca^{2+}-dependent PTH secretion to the right (6), therefore, FHH patients have higher PTH for their serum Ca^{2+} levels and they need to substantially increase their serum Ca^{2+} levels to suppress PTH secretion (7). Biochemical findings of patients with FHH include also very low urinary Ca^{2+} excretion and slightly elevated Mg_2+ levels. Serum concentrations of vitamin D metabolites are usually normal irrespective of whether PTH levels are inappropriately normal or mildly elevated (8). Although in most of the cases the condition is benign, patients with FHH may develop complications like gallstones or acute pancreatitis (9).

In this study, we present the functional significance of a CaSR mutation identified in an infant with FHH who presented with asymptomatic hypercalcemia detected early in the infantile period.

Case Report

Patient

A 4.5-month-old female infant of Greek origin was referred to our department in December of 2011 for evaluation of hypercalcemia detected in laboratory investigations performed during her hospital admission for urinary tract infection. She was born at term via normal vaginal delivery to healthy unrelated parents. She was growing well, her weight was at the 50th percentile, and her psychomotor development was normal. The biochemical evaluation confirmed her hypercalcemia (Ca^{2+}: 11.8 mg/dL; normal range: 9-11 mg/dL), accompanied by normal phosphorus (P: 5.9 mg/dL; normal range: 4-7 mg/dL), alkaline phosphatase (ALP: 360 U/L; normal range: 169-372 U/L), and albumin levels (Alb: 4.5 g/dL; normal range: 3.5-5 g/dL), while magnesium levels were slightly elevated (Mg_2+: 2.5 mg/dL; normal range: 1.4-1.7 mg/dL). The 24 h urinary Ca^{2+} excretion was reduced with a significantly low Ca^{2+} to creatinine clearance ratio (CCCR) (urine Ca^{2+}: 0.7 mg/kg/24 h; normal range: 2.8±0.66 and CCCR: 0.003; <0.01 suggests FHH, while >0.02 primary hyperparathyroidism). PTH levels were normal (PTH: 40 pg/mL; normal range: 10-80 pg/mL), while vitamin D levels were at the lowest range {25-hydroxyvitamin D [25(OH)D]: 21 ng/mL; normal range: 10-80 ng/mL}. Her mother's laboratory evaluation revealed severe hypovitaminosis D [25(OH)D: 4 ng/mL] with normal Ca^{2+} (9.7 mg/dL; normal range: 8.9-10.1 mg/dL), P (2.8 mg/dL; normal range: 2.3-4.7 mg/dL), ALP (68 U/L; normal range: 20-130 U/L), and PTH levels (40.5 pg/mL; normal range: 10-80 pg/mL). It is interesting to note that the infant was exclusively breastfed and did not receive any vitamin D supplementation. Her father's Ca^{2+} levels were at the upper normal range (Ca^{2+}: 10.3 mg/dL), with normal P (2.66 mg/dL) and ALP (85 U/L). With the suspicion of FHH, mutational analysis of the *CaSR* gene was performed.

Genetic Analysis

Genomic DNA was isolated from whole blood using the Purelink Genomic DNA kit (Invitrogen Ltd, UK). Exons 2-7 of *CaSR* gene (NM_000388) and their respective flanking regions were amplified, and polymerase chain reaction (PCR) products were sequenced on ABI 310 (Applied Biosystems, Foster City, CA, USA). Primers and PCR conditions are available upon request. Informed consent was obtained from the parents of the patient in order to pursuit the genetic analysis.

Site-Directed Mutagenesis

The human CaSR cDNA cloned in the pCR 3.1 plasmid (pCR3.1/hCaSR) (Invitrogen) was kindly provided by Dr. Lia Baldini, University of Limoges, France. The GTinsA mutation was directly introduced to the wild-type (WT) CaSR plasmid to generate the mutated receptor using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. The set of primers overlapping the target regions of the WT cDNA were: 5'-CAGCATGTGGTAGAGAGATTCAAAATTCCAC-3' and 5'- GTGGAATTTTGAATCTCTCTACCACATGCTG-3'.

Cell Culture

Human embryonic kidney (HEK) 293 T cells were cultured in Dulbecco's modified Eagle's medium (Sigma; MO, USA) containing 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL), and 2 mM L-glutamine in a

humidified incubator at 37 ºC and a steady supply of 5% CO_2. The cells were transiently transfected with CaSR cDNA (12 μg) in 10 cm tissue culture plates, using calcium phosphate DNA precipitates. Ca^{2+} measurements were performed 60 h post transfection.

Free Intracellular Ca^{2+} [$(Ca^{2+})_i$] Measurements

Sixty hours post transfection, cells were loaded with Fura 2-AM as already described (10). Cell pellets were suspended in Krebs-Ringer-HEPES (KRH) buffer [125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 6 mM D-glucose, and 25 mM HEPES-NaOH (pH 7.4)] containing 2% FBS counted in a hemocytometer and loaded with Fura 2-AM dye (5.0 μM), for 30 min at RT in the dark. Following loading, the cells were washed twice with $CaCl_2$- deprived KRH and re-suspended in $CaCl_2$-free KRH supplemented with 250 mM sulfinpyrazone to prevent dye leakage. Cell aliquots ($1.5x10^6$) were transferred to a thermostatted cuvette (37 ºC), maintained under continuous stirring, and analyzed in a Perkin-Elmer LS-55. The basal levels of $(Ca^{2+})_i$ were measured upon addition of 1 mM EGTA and presented as nM. To investigate alterations in CaSR function, $CaCl_2$ (3 mM) was reintroduced into the medium, and $(Ca^{2+})_i$ increase was recorded.

Statistical Analysis

All data are expressed as mean ± standard error of mean. Statistical analysis for multiple comparisons was performed using a one-way analysis of variance (ANOVA) followed by Tukey honest significant difference post-hoc test. Non-directional student's t-tests were performed for comparisons involving only two groups. All statistical analyses were conducted using the Graph-Pad Prism software. Results were considered statistically significant at $p \leq 0.05$.

Identification of the Calcium-Sensing Receptor Mutation

Direct sequencing of the coding region of *CaSR* gene revealed the heterozygous mutation c.772_773delGTinsA in exon 4 resulting in the substitution of amino acid valine (Val) with amino acid arginine (Arg) and the premature pause of the translation 46 amino acids later (p.Val258ArgfsTer47) (Figure 1). The same mutation was detected in the father but not in the mother. This change was not found in 50 unrelated individuals of the general population.

Functional Analysis

As already described, the WT and the mutant CaSR were transiently expressed in cultured HEK 293 T cells. The induction of $(Ca^{+2})_i$ influx for both the WT and the mutant CaSR, was estimated after reintroduction of 3 mM $CaCl_2$ (3 mM) into the medium. $[Ca^{+2}]_i$ increase was recorded as the net nM difference between the peak of $(Ca^{+2})_i$ and the base-line $(Ca^{+2})_i$ measurements. In Figure 2, it is shown that the WT over-expressing cells present a threshold of $(Ca^{2+})_i$ of 118.6±5.590 nM. The cells harboring the c.772_773delGTinsA mutation exhibit significantly lower $(Ca^{2+})_i$ (71.42±3.977 nM), $p<0.01$. Moreover, stimulation with exogenous addition of $CaCl_2$ resulted in a significantly poorer response of the c.772_773delGTinsA over-expressing cells (17.33±0.7028 ΔnM) in comparison to WT (27.77±3.870 ΔnM), $p<0.05$.

Discussion

In this report, we studied the functional consequences of a CaSR mutation identified in an infant with mild hypercalcemia, admitted in our Pediatric Department. The genetic change was located in exon 4 of the *CaSR* gene and resulted in a new reading frame and theoretically, in a shorter CaSR protein. The same genetic change has been recently described in an adult patient with mild hypercalcemia and her two siblings originating

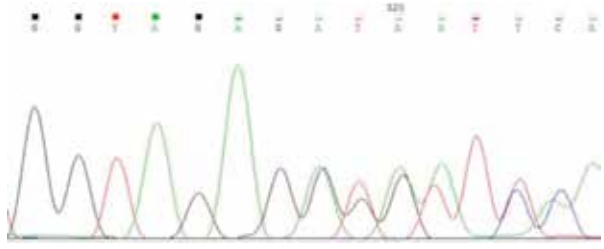

Figure 1. Sequencing analysis of *CaSR* gene (exon 4). Detection of c.772_773delGTinsA mutation in our proband

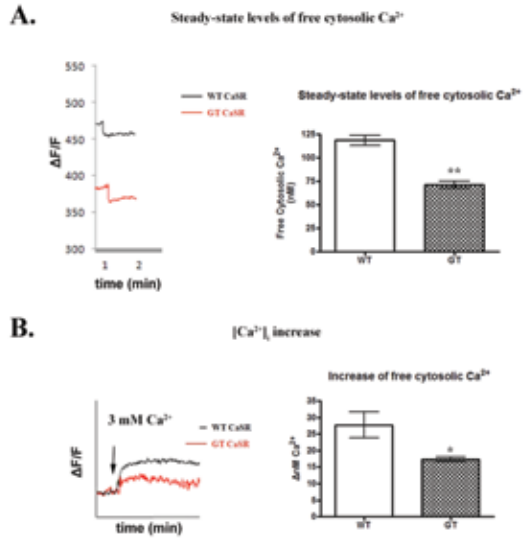

Figure 2. Alterations of Ca^{2+}-evoked responses in the presence of CaSR mutation: (A) Steady-state levels of free cytosolic Ca^{2+}. Representative Ca^{2+} traces are shown in the left panel and quantitative analysis of the $(Ca^{2+})_i$ is depicted on the right panel. n=3; **p<0.01, comparing between wild-type and GT, using unpaired t-test. (B) Ca^{2+} influx upon stimulation with $CaCl_2$. Representative Ca^{2+} measurements are shown in the left panel and quantitative estimation of $(Ca^{2+})_i$ increase (ΔnM), upon $CaCl_2$ stimulation is depicted on the right panel. n=3; *p<0.05, comparing between wild-type and GT using unpaired t-test. WT: wild-type

from a Greek island (Nisyros) (11), however a functional assay was not performed to clarify the functional impact. No common ancestry is known between our patient and the patient from Nisyros.

The functional analysis showed that the mutant CaSR transfected into HEK 293 cells led to a statistically significant decrease of $(Ca^{2+})_i$ influx after stimulation with exogenous Ca^{2+} compared with the WT. According to the known intracellular signaling pathway of CaSR, in response to binding of ligands to the receptor, the TMDs couple to G proteins and elicit several intracellular signaling cascades (12). These involve the activation of phospholipase C (PLC), breakdown of phosphatidylinositol 4,5-biphosphate, and formation of inositol 1,4,5-triphosphate (IP3) (13). Hence, high extracellular Ca^{2+} concentration stimulates the release of $(Ca^{2+})_i$ from the intracellular stores which acts as a secondary messenger with different cell-dependent actions (14). Given that our mutation is located on the extracellular domain of the protein leading to a protein that lacks part of the ECD, the entire TMDs and the ICD possibly affect both ligand binding and cell signaling functions.

Approximately 130 inactivating mutations have been identified in patients with FHH, including mainly missense mutations. Most of them are clustered in the N-terminal domain and may alter the function of the receptor by affecting either the ligand binding or the G protein coupling (15). To our knowledge, 8 nonsense CaSR mutations leading to a shorter CaSR protein have been reported in the literature. These mutations have been described in exons 2 (1 mutation), 4 (2), 6 (1), and 7 (4) and are localized mainly in the ECD, while two mutations were found in the intracellular loop (ICL) [www.casrdb.mcgill.ca]. As these mutations usually lead to a severely truncated protein, one would expect to result in a more severe phenotype. However, in most of the reported cases, patients feature typical findings with mildly elevated Ca^{2+} (16,17) as was also observed in our case and her father as well as in the patients from Nisyros (11). Previous research has suggested that alterations caused by specific mutations depend on the location of the substituted amino acid in one of the critical regions for ligand binding, receptor trafficking, and signal transmission but not on the extend of the defect (13). Possible mechanisms by which inactivating mutations alter CaSR function include: incorrect folding and cellular trafficking of the protein thus resulting in reduced cell surface expression of the receptor (18); disruption of the disulfide bridge between ECL1 and ECL2 leading to abnormal secondary structure; abnormal conformational changes that follow ligand binding thus affecting G-protein coupling (19). It is also evident that missense mutations with a dominant negative effect, where the abnormal receptors dimerize with the wild types, result in a higher degree of hypercalcemia and a more severe phenotype than nonsense mutations producing considerably abnormal transcripts (16,17). This hypothesis can explain the mild phenotype observed in our patient although the identified CaSR mutation resulted in a truncated product of 303 aa instead of 1078. Unfortunately, we were not able to perform the Western blot analysis to further investigate the expression of CaSR.

Disorders of Ca^{2+} homeostasis featured by hypercalcemia are usually associated with hyperparathyroidism (HPT) either primary, secondary, or tertiary. The majority of cases of HPT are sporadic (95%), while only 5% are associated with a hereditary syndrome. Inheritable disorders include NSHPT, isolated familial HPT, multiple endocrine neoplasia syndromes (MEN-1, MEN-2A, MEN-4), HPT-jaw tumor syndrome, familial hypercalciuric hypercalcemia, and FHH (20). In the past years, patients with FHH misdiagnosed as primary HPT had inappropriately undergone parathyroidectomy without resolution of their symptoms (21). A recent consensus report of the European Society of Endocrine Surgeons clearly states that surgery is contraindicated in FHH, however, in some cases, the differential diagnosis from other conditions, where surgical management is necessary, can be challenging (22). In particular, the clinical picture of patients with FHH can be complicated by vitamin D deficiency which is widely prevalent. It is evident that vitamin D deficiency results in PTH elevation, and treatment is associated with normalization of PTH and lower Ca^{2+} levels (23). Our patient had insufficient vitamin D levels, while PTH levels did not exceed the normal range. Furthermore, although one of the typical features of FHH is hypocalciuria, some patients may present with hypercalciuria caused by the distinct functions of the mutated CaSR in renal and parathyroid cells leading to the incorrect diagnosis of primary HPT (24). Further, due to the tissue-specific CaSR-mediated signaling pathways, patients with FHH can manifest uncommon complications like pancreatitis, osteomalacia, and nephrolithiasis (24,25,26) that are not expected in this otherwise benign condition.

Hence, genetic evolution and the identification of mutations of the *CaSR* gene helped clinicians to distinguish FHH from other causes of HPT as reliable distinction is not always possible on clinical grounds. The aforementioned consensus report indicates that the diagnosis of hereditary HPT should be confirmed by genetic analysis and followed by genetic counseling of both patient and relatives. The diagnosis of FHH should be excluded in all patients before scheduling parathyroidectomy (22). However, as genetic analysis is expensive and not always easily available, strict selection criteria should be followed in order to avoid pitfalls and increase the sensitivity of CaSR mutational analysis (27).

Nevertheless, it is worth mentioning that CaSR mutations account for only 65% of FHH (28). Recently, germline inactivating mutations of *GNA11*, a gene encoding the α-subunit of a G protein coupled with CaSR during the pathway of signal transmission were found in patients with FHH type 2 (29). Moreover, mutations of *AP2S1* were identified in patients with FHH type 3, a gene that encodes the adaptor protein 2 σ-subunit that is involved in CaSR endocytosis (30). Both these genetic defects result in impaired CaSR signaling leading to a

similar clinical phenotype. Yet, as these mutations seem to be very rare (31), *CaSR* molecular analysis is considered to be an appropriate first step in the genetic evaluation of patients with features of FHH.

In the present study, we showed that the Val258ArgfsTer47 mutation in the *CaSR* gene identified in a young patient with hypercalcemia and her father resulted in a significant reduction of CaSR sensitization to extracellular Ca^{2+} leading to typical features of FHH. This is the second family of FHH reported from Greece and, interestingly, both families bear the same mutation. Molecular analysis of the *CaSR* gene, especially in patients who present early in life with hypocalciuric hypercalcemia, can establish the diagnosis of FHH and facilitate the subsequent clinical management.

Acknowledgments

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Ethics

Informed Consent: Informed consent was obtained from the parents of the patient in order to pursuit the genetic analysis.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Anna Papadopoulou, Anastasios Papadimitriou, Design: Anna Papadopoulou, Data Collection or Processing: Katerina Melachroinou, Christos Meristoudis, Tania Siahanidou, Analysis or Interpretation: Anna Papadopoulou, Anastasios Papadimitriou, Literature Search: Anna Papadopoulou, Evangelia Gole, Writing: Anna Papadopoulou, Evangelia Gole.

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Case Report

Fluoxetine-Induced Hypoglycaemia in a Patient with Congenital Hyperinsulinism on Lanreotide Therapy

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ABSTRACT

Antidepressant drugs are reported to cause alterations in blood glucose homeostasis in adults with diabetes mellitus. We report a patient with persistent congenital hyperinsulinism (CHI) who developed recurrent hypoglycaemia following fluoxetine therapy. This 15-year-old girl was initially managed with diazoxide therapy. She developed troublesome hypertrichosis, which affected her quality of life adversely. Diazoxide was then slowly weaned and stopped with the introduction of octreotide, to which she responded well. Subcutaneous lanreotide (long-acting somatostatin analogue) was subsequently commenced (30 mg, once monthly) as injecting octreotide multiple times a day was proving to be difficult for the patient. The continuous blood glucose monitoring on monthly lanreotide injections revealed good glycaemic control. Six months later, she developed depression due to psychosocial problems at school. She was started on fluoxetine by the psychiatry team. She subsequently developed recurrent symptomatic hypoglycaemic episodes (blood glucose <3.5 mmol/L) and fluoxetine was discontinued, following which the hypoglycaemic episodes resolved within a week. Fluoxetine has been associated with hypoglycaemia in patients with diabetes mellitus. We report, for the first time, hypoglycaemia secondary to fluoxetine in a patient with CHI.

Keywords: Fluoxetine, selective serotonin reuptake inhibitor, hypoglycaemia, hyperinsulinism

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Diazoxide is used as the first-line medication in congenital hyperinsulinism (CHI). Troublesome hypertrichosis may occur as a side-effect which may lead to depression, particularly in adolescent females. Fluoxetine, an antidepressant, has been implicated to cause disturbances in glucose homeostasis in patients with diabetes.

WHAT THIS STUDY ADDS?

Fluoxetine can cause or worsen hypoglycaemia in patients with CHI. This has not been reported in the literature before.

Introduction

Congenital hyperinsulinism (CHI) is a disorder caused by dysregulated insulin secretion from the beta cells of the pancreas and is the major cause of hypoglycaemia during infancy and childhood (1). Antidepressant agents have been implicated to contribute to glucose dysregulation, causing both hypo- and hyperglycemia, depending on the specific medication used (2). Such disturbances have been reported in normal

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subjects as well as in patients with diabetes mellitus (DM). Selective serotonin reuptake inhibitors (SSRIs), in particular fluoxetine, have been associated with hypoglycaemia, reduced awareness of hypoglycaemic episodes, and increased insulin sensitivity in patients with DM (3,4,5,6,7). It is well known that unrecognised and untreated hypoglycaemia can be devastating and can potentially cause neurological damage. Moreover, the hypoglycaemic episodes occurring as a side-effect of these medications can have a negative psychological impact in a chronic condition such as DM and persistent forms of CHI. It is therefore important to be aware of the potential impact of antidepressants on glycaemic control in patients with DM and CHI. Fluoxetine-induced hypoglycaemia has been reported in the literature in adult patients with DM with paucity of literature in the paediatric population. Also, to our knowledge, there are no previous published report of fluoxetine-induced hypoglycaemia in patients with CHI. We report a 15-year-old girl with CHI who developed persistent and recurrent hypoglycaemia secondary to fluoxetine.

Case Report

A 15-year-old girl with a diagnosis of CHI was referred to our clinic from a District General Hospital. She was born to healthy non-consanguineous Caucasian parents. She was noted to be hypoglycemic since birth and subsequent diagnostic work up for hypoglycaemia led to the diagnosis of CHI. CHI was defined as an inappropriately elevated insulin level (100 pmol/L) during hypoglycaemia (2 mmol/L) with suppressed fatty acids (<100 µmol/L) and 3-hydroxy butyrate (<100 µmol/L). Genetic analysis of the patient revealed a *de novo* heterozygous ABCC8 mutation. She was started on diazoxide (5 mg/kg/day), in conjunction with chlorothiazide (7 mg/kg/day), to which she responded well. Chlorothiazide was subsequently weaned and stopped when the patient was 5 years of age. At this time, 18-Fluro DOPA positron emission tomography (PET) computed tomography scan of the pancreas revealed diffuse disease. While glycemic control was optimal on diazoxide, its long-term use caused hypertrichosis, a well-known side effect of the drug. The troublesome hypertrichosis continued through her teenage years and had a significant impact on her quality of life. She suffered from depression and was missing a lot of school. She was referred for therapies including wax therapy and laser to help with her hirsutism. Unfortunately, the hypertrichosis and hirsutism were not amenable to these therapies. This was imposing a negative impact on her quality of life with her committing deliberate self-harm on a few occasions. The patient was in continuous need for psychological assistance and support. She was electively admitted to our pediatric inpatient unit to decide on an alternative medical treatment for CHI. A trial off diazoxide was performed whereby, diazoxide was gradually weaned and stopped for 72 hours. However, this led to subsequent development of recurrent symptomatic hypoglycaemia (blood glucose <3.5 mmol/L). Further investigations revealed a persistent hyperinsulinaemic hypoglycaemia with a plasma insulin concentration of 96 pmol/L and a suppressed plasma ketone level (free fatty acids-608 µmol/L, 3 hydroxy butyrate-47 µmol/L) when the plasma blood glucose was 2.6 mmol/L. Octreotide (a somatostatin analogue) was then commenced as four times daily subcutaneous injection. Baseline investigations including ultrasound of gall bladder, thyroid function tests, and insulin-like growth factor-1 (IGF1) level prior to commencement of octreotide were within normal limits. Octreotide was started at 5 mcg/kg/day and was gradually built up to 15 mcg/kg/day as 4 divided subcutaneous injections. A good glycemic response was noted at this dose and she was able to tolerate a fast for a period of 24 hours with no evidence of hypoglycaemia. However, a 4 times daily injection therapy was becoming too labour intense for her and she did not tolerate this intense therapy. Hence, lanreotide (long-acting somatostatin analogue) was commenced as a subcutaneous injection 30 mg once monthly and octreotide was gradually weaned and stopped. The continuous blood glucose monitoring system following the administration of lanreotide revealed good glycaemic control with no episodes of hypoglycaemia. She also noted significant improvement in her quality of life and her school attendance improved.

Six months later, she developed depression due to psychosocial problems at school. She was assessed by a psychiatrist and was commenced on fluoxetine at a dose of 20 mg once daily. A week later, after the commencement of fluoxetine, the patient developed recurrent symptomatic hypoglycaemic episodes (blood glucose <3.5 mmol/L). The blood test during one of these hypoglycaemic episodes showed suppressed plasma insulin (<14 pmol/L) and c-peptide (<100 pmol/L) concentrations. Continuous blood glucose monitoring (CGM) was performed while the patient was on fluoxetine. The CGM did reveal multiple hypoglycaemic episodes (blood glucose <3.5 mmol/L) (Figure 1). After discussing with the psychiatric team, fluoxetine was discontinued, following which the hypoglycaemic episodes resolved within a week. CGM, two weeks after the discontinuation of fluoxetine, revealed a resolution of the hypoglycaemic episodes (Figure 2). The patient's depressive symptoms slowly improved over time with the help of counselling.

Discussion

Persistent CHI and DM are chronic medical conditions that cause disturbances in glucose homeostasis. DM is one of the commonest chronic diseases in childhood. Hypoglycaemia is a common occurrence in both these conditions. Moreover, chronic illnesses usually carry the risk of associated depression. For instance, in adults with DM, the risk of co-morbid depression is 8.5% to 20.0% higher than the general population (8). Treatment

of depression associated with chronic illness is important as delay or non-treatment may lead to poor glycaemic control. For instance, in patients with DM, depression is associated with hyperglycaemia and an increased risk for diabetic complications and treatment of depression is associated with improved glycaemic control (9). In our patient, the extent of psychosocial problems at her school was affecting her adversely and warranted the need for psychiatric counselling and antidepressant therapy.

SSRIs are one of the most commonly used antidepressant medications and are usually the first choice for depression because of fewer side effects than most other types of antidepressants (10). Fluoxetine, an SSRI, is an antidepressant that leads to enhanced serotonergic neurotransmission. Fluoxetine blocks serotonin reuptake and increases serotonin stimulation of receptors during the acute phase, and chronic use leads to desensitization of somatodendritic 5-HT1A and of terminal autoreceptors with an overall clinical effect of increased mood and decreased anxiety (11). Fluoxetine is indicated for use in pediatric patients over 8 years of age with multiple mental health diagnoses, including depression. It is a daily oral tablet, which can be titrated according to effect (12).

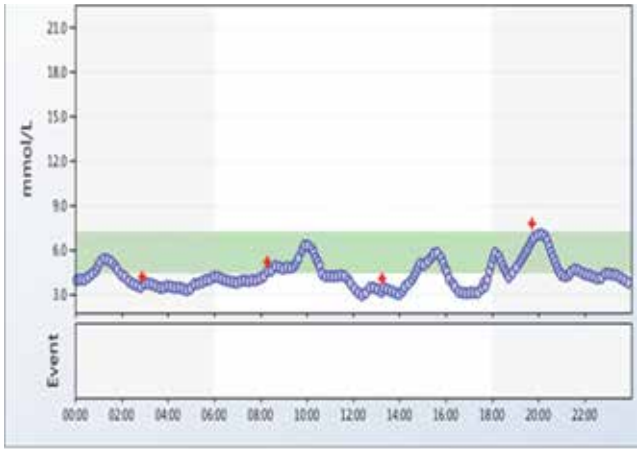

Figure 1. Continuous blood glucose monitoring on fluoxetine

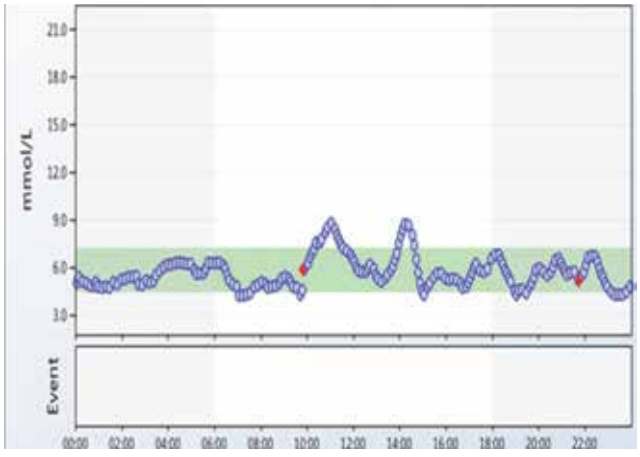

Figure 2. Continuous blood glucose monitoring off fluoxetine

In our patient, diazoxide was providing optimal management for hypoglycaemia but due to significant hypertrichosis interfering with lifestyle and not amenable to therapies, octreotide was introduced which proved to be effective. As octreotide involves multiple injections which were proving to be difficult for the patient, lanreotide (long-acting somatostatin analogue, 30 mg as subcutaneous injection, once a month) was introduced and was providing optimal glycaemic control (13), until our patient developed hypoglycaemic episodes following fluoxetine therapy.

Antidepressant drugs, particularly SSRIs, are reported to have a variety of effects on glucose homeostasis. SSRIs may cause hypoglycaemic unawareness secondary to autonomic dysfunction (14). Various mechanisms have been hypothesised to explain fluoxetine-induced hypoglycaemia. PET in healthy subjects has shown a decrease in the relative cerebral glucose metabolism in the amygdaloid complex, hippocampal formation, and ventral striatum with fluoxetine when compared to a placebo (15). McIntyre et al (2) suggested a central mechanism of action of fluoxetine. Potter van Loon et al (7) have shown that fluoxetine improves peripheral and hepatic insulin action in obese insulin-resistant subjects irrespective of its weight lowering effect. In a randomized, double-blind, placebo-controlled trial, insulin-mediated glucose disposal was measured in 12 obese patients with non-insulin dependent DM (NIDDM) on diet alone before and after four weeks of treatment with either placebo or fluoxetine and it was found that fluoxetine improves insulin-mediated glucose disposal in obese patients with NIDDM (6). These studies suggest that fluoxetine may have a role in increasing the insulin sensitivity. In our patient with CHI, a condition that causes an excessive and dysregulated insulin secretion, we believe that the introduction of fluoxetine has caused an increase in insulin sensitivity thereby resulting in recurrent hypoglycaemic episodes. Resolution of the hypoglycaemic episodes two weeks after stopping of fluoxetine substantiates this hypothesis.

In conclusion, this case demonstrates that hypertrichosis, a common side effect of diazoxide therapy for CHI, can result in depression, particularly in adolescent females. Management of diazoxide-responsive patients using long-acting somatostatin analogue, lanreotide, might be beneficial both in terms of increasing the patient's compliance and avoiding the side effects which usually constitutes the main concern for families and patients. Although fluoxetine-related hypoglycaemia has been reported in patients with DM, it may not be a widely recognized phenomenon among many health professionals. We report, for the first time, hypoglycaemia secondary to fluoxetine in a patient with CHI. We suggest that close blood glucose monitoring should be undertaken in patients with disorders of glucose homeostasis who are commenced on antidepressant therapy.

Ethics

Informed Consent: It was taken.
Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Dinesh Giri, Senthil Senniappan, Victoria Price, Mohammed Didi, Zoe Yung, Design: Dinesh Giri, Senthil Senniappan, Victoria Price, Mohammed Didi, Data Collection or Processing: Dinesh Giri, Zoe Yung, Analysis or Interpretation: Dinesh Giri, Zoe Yung, Senthil Senniappan, Literature Research: Dinesh Giri, Victoria Price, Senthil Senniappan, Writing: Dinesh Giri, Victoria Price, Senthil Senniappan.

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Case Report

Gonadoblastoma and Papillary Tubal Hyperplasia in Ovotesticular Disorder of Sexual Development

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ABSTRACT

Ovotesticular disorder of sexual development (DSD), formerly known as true *hermaphroditism*, is a rare form of DSD in which both testicular and ovarian tissues are present in the same individual either in a single gonad (ovotestis) or in opposite gonads with a testis and an ovary on each side. The diagnosis of ovotesticular DSD is based solely on the presence of ovarian and testicular tissue in the gonad and not on the characteristics of the internal and external genitalia, even if ambiguous. Herein, we report two patients with ovotesticular DSD-one presenting with ambiguous genitalia on the third day after birth and the other with short stature and primary amenorrhea in adolescence. Clinical and histopathological investigation revealed a sex-determining region on the Y chromosome (SRY)-positive 46,XX karyotype and bilateral ovotestes in case 1 and a 46,XY karyotype with hypergonadotropic hypogonadism and a streak gonad in one ovotestis with dysgerminoma, gonadoblastoma, and papillary tubal hyperplasia in the contralateral ovotestis in case 2. Laparoscopic examination and gonadal biopsy for histopathological diagnosis remain the cornerstones for a diagnosis of ovotesticular DSD. Moreover, SRY positivity in a 46,XX patient, a 46,XY karyotype, an intra-abdominal gonad, and the age of patient at the time of diagnosis are predictive risk factors for the development of gonadoblastoma and/or dysgerminoma in ovotesticular DSD.

Keywords: Ovotestes, sex-determining region on the Y chromosome, gonadoblastoma, dysgerminoma, tubal hyperplasia

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Ovotesticular disorder of sexual development (DSD) is a rare form of DSD. Delayed diagnosis and gonadectomy are the main reasons for development of gonadal malignancy in case of ovotesticular DSD.

WHAT THIS STUDY ADDS?

We report the first case of papillary tubal hyperplasia associated with gonadoblastoma and dysgerminoma in a case of ovotesticular DSD.

Introduction

Ovotesticular disorder of sexual development (DSD) can be diagnosed based only on histological criteria by detection of presence of both ovarian (containing follicles) and testicular tissue in the same gonad (ovotestis) or by the morphological appearance of the contralateral gonad. Approximately 60% of such

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patients have a 46,XX karyotype, 33% have a 46,XX/46,XY sex chromosome mosaicism, while the remaining 7% of patients have a 46,XY karyotype (1). Rarely, a sex-determining region on the Y chromosome (SRY) mutation may be identified in 46,XY ovotesticular DSD (2). On the other hand, SRY is present in only 10% of ovotesticular DSD cases with a 46,XX karyotype (3,4,5,6). The testes or testicular components of the ovotestes in ovotesticular DSD are likely to be dysgenetic, and gonadal tumors with a potential for malignancy occur in 2.6% of all cases (7). Dysgerminomas, gonadoblastomas, seminomas, and yolk sac carcinomas have been reported in ovotesticular DSD (8,9). Here, additional dysgerminoma and gonadoblastoma in a patient with 46,XY ovotesticular DSD and SRY positivity in a patient with 46,XX ovotesticular DSD are reported.

In this study, we report two cases of ovotesticular DSD for three reasons. Firstly, we wished to emphasize that cases of ovotesticular DSD may present at any age. Early diagnosis and prophylactic gonadectomy of ovotestesticular gonads can prevent the diagnostic delay for gonadoblastoma and/or dysgerminoma. Secondly, we wanted to show that pelvic ultrasound examination compared with laparoscopic examination has a relatively low sensitivity and specificity for establishing the presence or absence of gonads and Müllerian or Wolffian derivatives in DSD patients. Finally, to the best of our knowledge, we report the first case of papillary tubal hyperplasia (PTH) associated with gonadoblastoma and dysgerminoma in a case of ovotesticular DSD.

Case Reports

Case 1

The first patient was referred to our institution on the third day after birth due to ambiguous genitalia. The patient was born at term after a normal pregnancy with a birth weight of 3250 g, a body length of 51 cm, and a 35.5-cm head circumference and was the first child of unrelated parents. Except for this case, the medical history of the family was normal. Upon physical examination, the patient was found to have ambiguous genitalia (Figure 1) including a phallus with a length of 2.3 cm, bifid labioscrotal folds, incomplete labioscrotal fusion (between Prader stages II and III), a ventral opening of the urethra and chordae. The gonads were non-palpable bilaterally. In addition, there was no genital skin pigmentation. The initial workup was negative for congenital adrenal hyperplasia (CAH) and included measurements of 17-hydroxyprogesterone (85 ng/dL; normal 7-77 ng/dL), 11-deoxycortisol (27 ng/dL; normal 13-147 ng/dL), Δ4 androstenedione (62 ng/dL, normal 20-290 ng/dL), and dehydroepiandrosterone sulfate (59.7 µg/dL, normal 15-120 µg/dL). Additional hormone analysis revealed a follicle-stimulating hormone (FSH) level of 0.8 mIU/mL (normal, none to 5.5 mIU/mL), a luteinising hormone (LH) level of 0.1 mIU/mL (normal 0.02-0.3 mIU/mL), a total testosterone (T) level of 205 ng/dL (normal 20-187 ng/dL), an estradiol (E2) level of 26 pg/mL, and an anti-Müllerian hormone (AMH) level of 10 ng/mL (normal reference values for <1-year-old males 101.9-262.0 ng/mL, for <14-year-old females 0.3-11.2 ng/mL). Chromosomal analysis and fluorescence *in situ* hybridisation (FISH) of SRY revealed a SRY-positive 46,XX karyotype. A human chorionic gonadotropin (hCG; Pregnyl® 1500 U) test was performed to investigate presence of functional testicular tissue in the abdomen and testosterone synthesis defects. The test protocol included collection of a baseline blood sample (before the first injection of hCG between 8:00 and 9:00 a.m. on day 1) and collection of a second blood sample 24 h after the third injection of hCG to measure total T, dihydrotestosterone (DHT), and Δ4 androstenedione levels. Immediately following collection of the baseline blood sample, hCG (1500 U/1.73 m^2/dose) was administered intramuscularly for three days. Basal total T was 192 ng/dL; DHT, 26 ng/dL (normal, 16-79 ng/dL); Δ4 androstenedione, 38 ng/dL (normal, 5-45 ng/dL); and T/DHT ratio 7.4 (normal, 11.1±4). Following three injections of hCG, T was 603 ng/dL; DHT, 57 ng/dL; Δ4 androstenedione, 57 ng/dL; and T/DHT ratio 10,6 (normal <20). The hCG test confirmed the presence of functional testes or testicular tissues in the undescended gonads. Pelvic ultrasonography (US) showed a bicornuate uterus, tuba uterina, and round ligaments; neither gonad could be identified in pelvic areas or inguinal channels. Using laparoscopic examination, Müllerian remnants were identified and consisted of a gonad, a fallopian tube adjacent to the gonad with a bilateral fimbriated end (Figure 2), and a bicornuate uterus. The gonads were deeply located in retrocolic areas. Bilateral longitudinal wedge gonadal biopsies were performed to complete the SRY-positive 46,XX

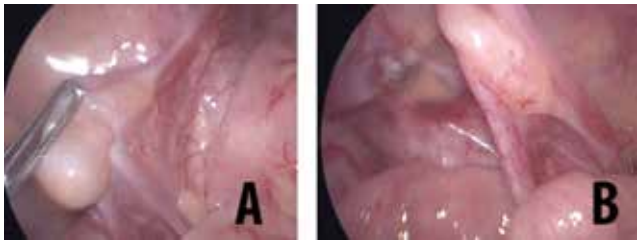

Figure 1. Appearance of external genitalia in case 1. There was a phallus 2.3-cm in length, bifid labioscrotal folds, absence of genital skin pigmentation (B), incomplete labioscrotal fusion (between Prader stages II and III), ventral opening urethra, and chordae (C)

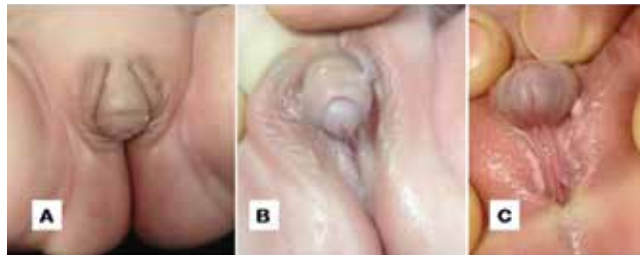

Figure 2. In case 1, appearance of the left (A) and right (B) gonads upon laparoscopic examination. The gonads were located in deep retrocolic areas

DSD evaluation. Histopathological examination of gonadal biopsies demonstrated features of bilateral ovotestes (Figure 3). The parents were informed that patients with SRY-positive 46,XX DSD and ovotesticular gonadal structures have a high risk of the development of malignant gonadal tumours in the future. At age 6 months, the parents decided to raise the child as a female and gave permission for prophylactic gonadectomy. Bilateral gonadectomy was performed using the laparoscopic method.

Case 2

A 15-year-old female patient presented with absence of pubertal development, primary amenorrhoea and parental concern regarding severe growth retardation. She was the first child of non-consanguineous parents. The medical history of the family was noncontributory. The patient was born at term with a length of 50 cm and a weight of 3450 g; the neonatal period and infancy were uneventful. At physical examination, she was prepubertal with Tanner stage I breast development and stage III pubic hair development. Her height was 149 cm [-2.1 standard deviation (SD)] which is below the third percentile (mid-parental height; between the 10th and 25th percentiles), and her weight was 48 kg (-0.72 SD), i.e. between the 10th and 25th percentiles. Physical examination revealed a normal female external genitalia phenotype. Further physical examination was unremarkable although her bone age was delayed by 3.5 years (Greulich and Pyle method). A baseline blood screen (urea, electrolytes, calcium, phosphate, liver enzymes, and full blood count), screening for thyroid function, and screening for coeliac disease antibodies revealed normal values. Hormone assays revealed low oestradiol (<10 pg/mL; normal 21-85 pg/mL), high FSH (73.7 mIU/mL; normal 1.8-11.2 mIU/mL), and high LH (33 mIU/mL; normal 1.5-9.0 mIU/mL) levels, indicating hypergonadotropic hypogonadism. Pelvic US revealed a small uterus 4.7 cmx1.3 mm in diameter and hypoplastic gonads (left pelvic gonad: 15 x 12 mm; right pelvic streak gonad: 7x6 mm). Due to the presence of a hypoplastic Müllerian structure, streak gonad, short stature, and primary amenorrhoea in this patient with a female phenotype and hypergonadotropic hypogonadism, a tentative diagnosis of Turner syndrome was made. However, the characteristic physical stigmata of Turner syndrome were not present. Chromosomal analysis and FISH of SRY showed an SRY-positive 46,XY karyotype. These findings indicate that gonadal dysgenesis should be considered in this differential diagnosis. Due to the fact that an SRY-positive 46,XY genotype and rudimentary gonads are associated with a high risk of malignancy, the parents and patient were informed of the malignancy risk of dysgenetic gonads. A bilateral gonadectomy was carried out by the laparoscopic method. Histopathological examination of the excised left gonad revealed features of an ovotestis with both ovarian and testicular tissues present in addition to a gonadoblastoma on the base of dysgerminomas (Figure 4). Histopathological examination of the right rudimentary gonad revealed streak gonad parenchyma with epididymis and papillary tuba hyperplasia (PTH; Figure 4D). Laparoscopic examination revealed a left tubular structure arising from the rudimentary uterus that ended with the left gonad (Figure 5), and a streak right gonad.

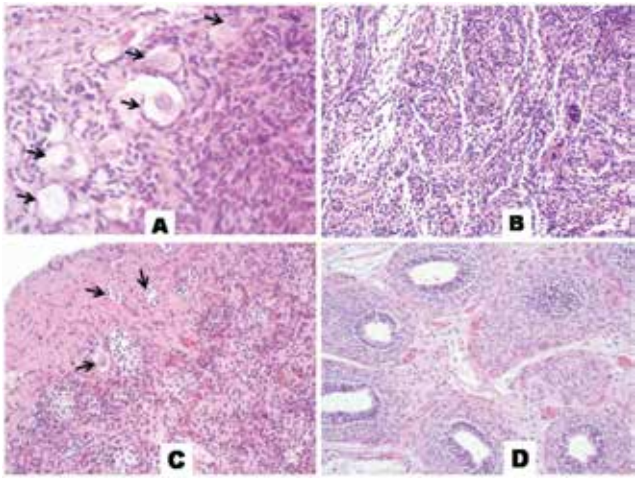

Figure 3. Histological examination of gonadal biopsy samples for case 1 revealed features of ovotestes which are characterised by ovarian stroma (H&E, x400) (A), and disorganised seminiferous tubules (H&E, x200) (B), right gonad. The ovarian follicles are seen on the left (arrows) surrounded by numerous small seminiferous tubules with immature Sertoli cells (H&E, x20). (C) The epididymis is lined by tall columnar cells, left side (H&E, x20) (D), left gonad

Discussion

Ovotesticular DSD is associated with heterogeneous clinical, genetic, and pathological spectra. Patients often

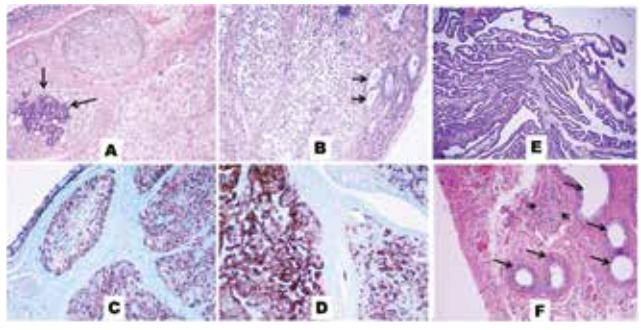

Figure 4. Histological examination of gonadoblastoma with superimposed dysgerminoma in the left gonad (A-D) and streak right gonad (E, F). (A) A focus calcification (arrows) lies in a dysgerminoma nest and tumour nests were encapsulated by immature granulose/Sertoli cells (middle upper) and were progressing to gonadoblastoma (H&E, x200). (B) Sertoli cells (arrows) and dysgerminoma nests progressing to gonadoblastoma (H&E, x400). Immunohistochemically, dysgerminoma cells showed reactivity with placental alkaline phosphatase (PLAP) (PLAP x400) (C) and c-kit (CD117) (CD117 x400) (D). (E) The right streak gonad showed polypoid and papillary hyperplasia of the tubular epithelium. Multiple small papillae floating in the tubal lumen (H&E, x200). (F) Leydig cell remnants (arrowheads) and epididymis (arrows) (H&E, x200)

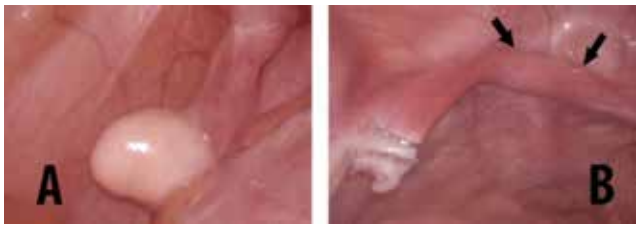

Figure 5. Laparoscopic images of the inner genital structures of case 2. (A) Tubular structure arising from the uterus ended with the left gonad. (B) Endoclips following a left gonadectomy and rudimentary uterus (arrows)

present in the neonatal period with ambiguous genitalia, as in case 1. However, patients may present with a normal female phenotype in adolescence or adult life, as in case 2. Investigation of the aetiology of DSD should be managed in a tertiary centre by a multidisciplinary team that includes a geneticist, a pediatric urologist or pediatric surgeon, and a pediatric endocrinologist.

Many excellent algorithms for the investigation of the aetiology of DSD are available. Palpable or non-palpable gonads, genital skin pigmentation, electrolytes, patient age at presentation, and family history are key clinical issues at initial evaluation. One of the current cases presented with ambiguous genitalia, non-palpable gonads, no genital skin pigmentation, and normal electrolytes. Many of these findings are inconsistent with a diagnosis of CAH, however, clinicians should rule out the diagnosis of CAH with an additional hormonal analysis. Here, the diagnosis of CAH was excluded by basal hormonal analysis. The second case presented with a normal female phenotype at the age of 15 years with short stature, primary amenorrhoea, and hypergonadotropic hypogonadism. In both cases, the next stage of diagnostic investigation consisted of SRY and chromosomal analyses. Case 1 exhibited an SRY-positive 46,XX karyotype and case 2 exhibited a SRY-positive 46,XY karyotype. These findings ruled out Turner syndrome in case 2. At this point, a diagnosis of gonadal dysgenesis as the aetiology of the SRY-positive 46,XX and 46,XY DSD cases was very close. The last step was the imaging of Müllerian and/or Wolffian derivatives, undescended gonads, and gonadal biopsy and/or gonadectomy for histopathological diagnosis.

The uterus and ovaries are relatively easy to find during the neonatal period using pelvic US, since these structures are prominent under the influence of maternal hormones (10). On the other hand, many studies discourage the use of pelvic US as the primary modality for establishing the presence or absence of gonads. Cohen et al (11) reported that only one ovary is identified in 40% of typical patients and none in 16%. Steven et al (12) was the first to assess the reliability of pelvic US in the evaluation of Müllerian derivatives in children with DSD and reported significant limitations, because pelvic US has only 54% sensitivity and 50% specificity. A recent meta-analysis of 12 studies (591 testes) indicated that pelvic US has an overall sensitivity and specificity of 44% and 95%, respectively (13). In the present study, neither gonad could be identified by pelvic US in one of the two cases; however, laparoscopic investigation revealed both gonads located deep in retrocolic areas. An additional advantage of laparoscopic investigation is the option to obtain a gonadal biopsy, as in case 1, or to perform a gonadectomy during the laparoscopic examination, as in case 2.

Informative predictive parameters for the development of gonadal type 2 germ cell tumours are the anatomical position of the gonad, gonadal differentiation, and the presence of a specific part of the Y chromosome (14). The majority of patients with ovotesticular DSD have 46,XX karyotypes (60%), while the remaining patients have 46,XY (12%) or mosaic karyotypes (28%) (1,15). SRY is present in only 10% of 46,XX ovotesticular DSD patients (3,4,5,6). The presence of SRY in a patient with 46,XX ovotesticular DSD strongly suggests that the ovotesticular gonads carry a high potential for malignancy, especially dysgerminoma (7,14). On the other hand, gonadoblastomas have been reported to be rarely present in patients who have a normal 46,XX karyotype (16).

The gonadoblastoma locus (GBY) is the only oncogenic locus on the human Y chromosome. It is postulated to serve a normal function in the testis but could exert oncogenic effects in dysgenetic gonads of individuals with DSD (17). The development of a gonadoblastoma is dependent on the presence of part of the Y chromosome, known as the GBY region (15). One of the putative candidate genes for the involvement of this region is the testis-specific protein Y (TSPY) on the Y chromosome (18). It serves normal functions in male stem germ cell proliferation and differentiation (17). Ectopic expression and actions of *TSPY* gene in incompatible germ cells, such as those in dysgenetic or ovarian environments and dysfunctional testis, such as ovotesticular gonad, disrupt the normal cell cycle regulation and predispose the host cells to tumorigenesis. The encoded protein is found to be highly expressed in carcinoma *in situ* and gonadoblastoma (16). Ectopic expression of SRY, as a part of the Y chromosome in case 1 diagnosed with 46, XX ovotesticular DSD is the main risk factor for the development of gonadoblastoma. On the other hand, the second patient, diagnosed as a case of 46, XY DSD, has two well-known risk factors for the development of gonadoblastoma. One of them is presence of ovotesticular gonads and the second - the intra-abdominal localization of these gonads with testicular components for 15 years.

Histological examination of gonad biopsies in case 1 revealed bilateral ovotestes. Histological examination of excised gonads in case 2 revealed features of an ovotestis with a gonadoblastoma and dysgerminomas in the left gonad and streak gonad parenchyma and PTH in the right gonad. Gonadoblastoma and/or dysgerminomas in cases of ovotesticular DSD are well-known findings, however, PTH is a novel finding in ovotesticular DSD. Originally described by Kurman et al (19), PTH was identified in 20 (91%) of 22 patients with ovarian non-invasive low-grade serous tumours. According to this description, PTH is the most-advanced stage of tubal hyperplasia and characterised by tubal proliferations that exhibit papillary tufting and detached clusters of bland

epithelium. These clusters of epithelial cells and small papillae are found floating in the lumen or protruding from the tubal mucosa into the lumen. The authors concluded that PTH is likely the precursor lesion and the small papillae and clusters of cells from the fallopian tubes implant on ovarian and peritoneal surfaces resulting in generation of low-grade serous tumours. Robey and Silva (20) found PTH in 68.7% of patients with ovarian serous borderline tumours. To our knowledge, this is the first report of PTH associated with ovotesticular DSD. PTH may be another tumour precursor in ovotesticular DSD.

In conclusion, in patients with gonadal dysgenesis or ovotesticular DSD, laparoscopic examination is the most sensitive and specific imaging modality for the evaluation of Müllerian derivatives and undescended gonads. This procedure has additional advantages, such as the potential to perform a gonadal biopsy for histopathological diagnosis or a gonadectomy to prevent or treat malignancy development. The presence or absence of SRY should be routinely investigated in patients with DSD because if a patient with ovotesticular DSD has Y-chromosome or SRY positivity, ovotestes or streak gonads should be excised before the development of gonadal malignancy. Furthermore, if PTH is identified following a histopathological examination, rudimentary Müllerian structures should also be excised completely to prevent gonadal or peritoneal serous tumours.

Ethics

Informed Consent: Informed consent was obtained from the patient's parents to participate in the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Enver Şimşek, Çiğdem Binay, Design: Enver Şimşek, Baran Tokar, Data Collection or Processing: Enver Şimşek, Çiğdem Binay, Meliha Demiral, Sare Kabukçuoğlu, Melek Üstün, Analysis or Interpretation: Enver Şimşek, Baran Tokar, Sare Kabukçuoğlu, Literature Search: Enver Şimşek, Meliha Demiral, Writing: Enver Şimşek, Meliha Demiral.

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Case Report

Corticosterone Methyl Oxidase Deficiency Type 1 with Normokalemia in an Infant

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ABSTRACT

Isolated aldosterone synthase deficiency may result in life-threatening salt-wasting and failure to thrive. The condition involves hyperkalemia accompanying hyponatremia. Two types of aldosterone synthase deficiency may be observed depending on hormone levels: corticosterone methyl oxidase type 1 (CMO 1) and CMO 2. Herein, we describe a Turkish infant patient with aldosterone synthase deficiency who presented with failure to thrive and salt wasting but with normal potassium levels. Urinary steroid characteristics were compatible with CMO I deficiency. Diagnosis of aldosterone synthase deficiency was confirmed by mutational analysis of the *CYP11B2* gene which identified the patient as homozygous for two mutations: c.788T>A (p.Ile263Asn) and c.1157T>C (p.Val386Ala). Family genetic study revealed that the mother was heterozygous for c.788T>A and homozygous for c.1157T>C and the father was heterozygous for both c.788T>A and c.1157T>C. To the best of our knowledge, this is only the second Turkish case with a confirmed molecular basis of type 1 aldosterone synthase deficiency. This case is also significant in showing that spot urinary steroid analysis can assist with the diagnosis and that hyperkalemia is not necessarily part of the disease.

Keywords: Corticosterone methyl oxidase, salt wasting, *CYP11B2* gene, failure to thrive

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Isolated aldosterone synthase deficiency may result in life-threatening salt-wasting and failure to thrive. To the best of our knowledge, the literature contains only one case of a Turkish patient with aldosterone synthase deficiency with confirmed mutation in the *CYP11B2* gene.

WHAT THIS STUDY ADDS?

We described a Turkish patient with aldosterone synthase deficiency presenting with failure to thrive and salt-wasting but with normal potassium levels in infancy. Diagnosis of aldosterone synthase deficiency was confirmed by mutational analysis of the *CYP11B2* gene.

Introduction

Aldosterone is essential to life due to two important functions - sodium excretion and intravascular volume regulation. It is synthesized by aldosterone synthase and encoded by *CYP11B2* gene on the long arm of chromosome 8. Beginning from 11-deoxycorticosterone in the zona glomerulosa of the adrenal cortex, aldosterone synthase catalyzes the sequential activities of

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11β-hydroxylase, 18-hydroxylase, and, finally, 18-methyl oxidase, the final three steps in aldosterone synthesis (1,2). Classically, CYP11B2 was considered to utilize corticosterone generated from 11-deoxycorticosterone by action of 11β-hydroxylase (CYP11B1), but 11-deoxycorticosterone proved to be the better substrate for CYP11B2 (2).

In infancy, aldosterone synthase deficiency generally takes the form of a life-threatening electrolyte imbalance. Failure to thrive, vomiting, and severe dehydration are commonly observed in children with the condition. Two types of aldosterone synthase deficiency have been described: corticosterone methyl oxidase type 1 (CMO 1) and CMO 2. These conditions can be differentiated by the presence of insufficient or excessive 18-OH-corticosterone, respectively. CMO 1 is typically characterized by total suppression of aldosterone synthase with no detectable levels of aldosterone release. In contrast, some degree of aldosterone synthase activity persists in CMO 2 deficiency, and low to normal levels of aldosterone may be observed (3). This also explains why type 1 has a more severe course.

To the best of our knowledge, the literature contains only one case of a Turkish patient with aldosterone synthase deficiency with confirmed mutation in the *CYP11B2* gene (4). In this report, we describe the second Turkish patient with aldosterone synthase deficiency who presented in infancy with failure to thrive and salt wasting. Urinary steroid characteristics were compatible with CMO I deficiency. Subsequent molecular genetic analysis on the *CYP11B2* gene confirmed the diagnosis.

Case Report

Our patient was delivered normally at 38 weeks gestation with a birth weight of 3200 g and length of 50 cm. The parents are Turkish and non-consanguineous. The patient presented at the age of 2 months with vomiting and failure to thrive. On examination, his body weight was 3.800 g (3rd percentile) and length was 53 cm (3rd to 10th percentile). She had a normal female phenotype and no hyperpigmentation was noted. Blood pressure was 80/50 mmHg.

Laboratory examination revealed a plasma sodium level of 127 mmol/L (136-145), plasma potassium of 5.1 mmol/L (3.5-5.5), and normal blood urea nitrogen and plasma creatinine. Urinary sodium levels were elevated (80 mmol/L) despite hyponatremia. Hormonal evaluation revealed cortisol of 8 µg/dL (5-25) and adrenocorticotropic hormone (ACTH) of 9.6 pg/mL (10-55). Plasma renin activity was 128 ng/mL/h (2.4-37) and plasma aldosterone 28 pg/mL (50-900). Aldosterone synthase deficiency was suspected. The patient was started on fludrocortisone (0.05 mg/day) and intravenous normal saline and responded well to treatment.

A spot urine sample was obtained before treatment, the steroid profile of which was analyzed using gas chromatography-mass spectrometry (GC-MS). This was carried out according to our previously published method (5). In brief, steroids were extracted, and conjugates were hydrolysed enzymatically using Helix pomatia digestive juice. The free steroid products were then re-extracted, and methyloxime–trimethylsilyl ether (MO–TMS) derivatives were prepared before analysis by GC-MS using a Perkin Elmer Clarus 500 system with an OV-1 column (Perkin Elmer, Beaconsfield, Buckinghamshire, UK). The steroid metabolites present in greatest quantities are quantified based on data obtained in cyclic scan mode. These comprise metabolites of androstenedione, dehydroepiandrosterone, progesterone, 17-hydroxyprogesterone, corticosterone, and cortisol.

Our patient's urine steroid profile was typical of aldosterone synthase deficiency type 1 in that tetrahydroaldosterone, the major metabolite of aldosterone, was undetectable and corticosterone metabolites were elevated relative to cortisol metabolites, but there were no increases of 18-hydroxylated corticosterone metabolites (Table 1).

Genetic Study

Written informed consent was obtained from the patient's parents to participate in the study.

Polymerase Chain Reaction and DNA Sequencing

Genomic DNA was extracted from peripheral leukocytes. All coding regions of *CYP11B2* and the exon-intron splicing junction boundaries were amplified using the polymerase chain reaction method, followed by sequencing.

Sequence analysis of *CYP11B2* showed that the patient was a homozygous for c.788T>A (p.Ile263Asn) and c.1157T>C (p.Val386Ala) (Figure 1A, 1B). Family genetic study showed that the mother was heterozygous for c.788T>A and homozygous for c.1157T>C, while the father was heterozygous for both c.788T>A and c.1157T>C.

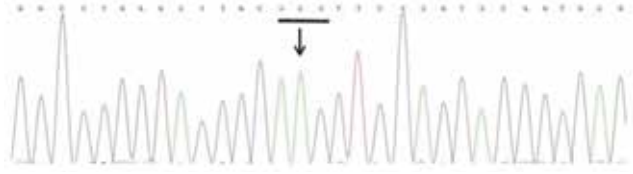

Figure 1A. Electropherogram of segment of the *CYP11B2* gene showing the mutation c.788T>A (p.Ile263Asn) in homozygous state in the patient. The affected codon is underlined

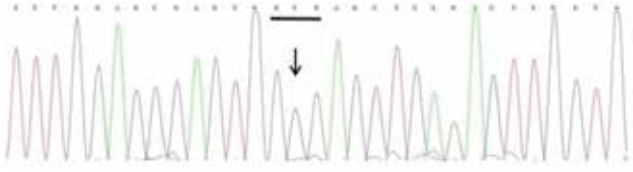

Figure 1B. Electropherogram of segment of the *CYP11B2* gene showing the mutation c.1157T>C (p.Val386Ala) in homozygous state in the patient. The affected codon is underlined

Table 1. Urinary steroid profiles

Urine steroid profiles	Patient μg/L	Reference values (mean ± SD)
THB	268	53±24
THA	84	118±39
allo-THB	287	184±54
THAld	Undetectable	
THE	305	702±256
THF	33	266±89
5αTHF	57	337±125
α-cortolone	16	193±85
β-cortolone+β-cortol	36	240±93
α-cortol	5	94±43
Ratio corticosterone metabolites/cortisol metabolites	1.41	0.20±0.1

THB: tetrahydrocorticosterone, THA: tetrahydro-11-dehydrocorticosterone, THAld: tetrahydroaldosterone, THE: tetrahydrocortisone, THF: tetrahydrocortisol, SD: standard deviation

Discussion

Aldosterone synthase deficiency may rarely be encountered as a cause of hyponatremia and failure to thrive in infants. When the disease is suspected, it is of vital importance that fludrocortisone therapy be initiated in addition to appropriate fluid replacement. The presence of hyperkalemia is not essential in order to establish the diagnosis. Normal potassium levels despite hyponatremia have been reported in some patients with aldosterone synthase deficiency, similar to our case (6). One recent experimental study investigated the mechanisms involved in renal control of potassium homeostasis in complete aldosterone deficiency. The results showed that renal adaptation to a physiological K (+) load in aldosterone deficiency is possible by means of aldosterone-independent activation of the renal outer medullary K (+) channel and epithelial sodium channel. Angiotensin II may also contribute to this (7).

It is difficult in infants to collect urine samples over a 24-hour period. Spot urinary steroid profiling is also useful tool to diagnose aldosterone synthase deficiency and other congenital adrenal diseases (8). Our patient's urinary steroid profile was analyzed with a spot urinary sample, with findings consistent with CMO 1 deficiency.

Cases of aldosterone synthase deficiency have been reported in various ethnic groups, including Europeans, North Americans, and Asians (9,10,11,12). One previous case of aldosterone synthase deficiency in a patient of Turkish origin was reported from Japan (4). Ours is the first Turkish patient to be genetically confirmed and reported from Turkey. Type 1 CMO was similarly present in the other Turkish patient. We were unable to obtain detailed clinical and laboratory information from the other Turkish patient for comparison with our own case, but the mutation in the other patient was different. We identified two homozygous mutations in *CYP11B2*, i.e. c.1157T>C and c.788T>A. Family genetic study showed that while the father was heterozygous for these two mutations, the mother was heterozygous for c.788T>A and homozygous for c.1157T>C.

In terms of the literature concerning the homozygous mutation, p.Val386Ala (c.1157T>C), the study of Pascoe et al (13) described the variant p.Val386Ala in Iranian-Jewish kindred previously, causing a small but reproducible reduction in the production of 18-hydroxycorticosterone *in vitro*. They hypothesized that the presence of another mutation is required if the gene is to become defective. Therefore, despite the mother is homozygous for p.Val386Ala, the overall genetic findings in her are still consistent with her being a carrier of aldosterone synthase deficiency. Since we were unable to study our patient's mother's urine steroid profile, we do not know whether subtle mineralocorticoid abnormalities were present. When we inquired into her medical history, including childhood, we learned that she had no marked adrenal insufficiency, had never undergone hyponatremia attack, and had not been hospitalized. However, there is a probability of mild mineralocorticoid deficiency in the mother's history. Another reason why the mother is today completely healthy may be that the condition gradually improves with declining renal tubular resistance to mineralocorticoids (3). Second homozygous non-synonymous variant in the patient is a novel c.788T>A (p.I263N) change which is also predicted to be disease-causing with a PolyPhen-2 score of 0.999 (http://genetics.bwh.harvard.edu/cgi-bin/pph2). This variant was neither found in ExAC nor in 1000 G.

In conclusion, this is the first report from Turkey of a Turkish patient with type 1 aldosterone synthase deficiency with a confirmed molecular basis and in which a spot urine steroid profile was used for making the diagnosis. Although aldosterone synthase deficiency is very rare, it is one of the diseases associated with hyponatremia and failure to thrive in infancy.

Ethics

Informed Consent: Written informed consent was obtained from the patient's parents to participate in the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Ala Üstyol, Mehmet Emre Atabek, Design: Ala Üstyol, Mehmet Emre Atabek, Data Collection or Processing: Ala Üstyol, Analysis or Interpretation: Norman Taylor, Matthew Chun-wing Yeung, Angel O. K. Chan, Literature Search: Ala Üstyol, Writing: Ala Üstyol.

Financial Disclosure: The authors declared that this study received no financial support.

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Case Report

Pyridoxine-Responsive Seizures in Infantile Hypophosphatasia and a Novel Homozygous Mutation in ALPL Gene

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ABSTRACT

Hypophosphatasia is a rare inherited disorder of bone and mineral metabolism caused by a number of loss-of-function mutations in the *ALPL* gene. It is characterized by defective bone and tooth mineralisation associated with low serum and bone alkaline phosphatase activity. The clinical presentation of this disease is extremely variable. For this reason, the diagnosis can be difficult and is often missed out or delayed. Hypophosphatasia is classified into subtypes based on the age of onset and clinical features. The clinical severity is associated with the age at diagnosis and the lack of tissue-nonspecific alkaline phosphatase activity; the severe forms of hypophosphatasia are primarily perinatal and infantile forms. Severe forms may present with many neurological problems such as seizures, hypotonia, irritability. Herein, we report the case of an infantile hypophosphatasia patient who presented with pyridoxine-responsive seizures and a novel homozygous mutation in the *ALPL* gene was detected. There is a limited number of hypophosphatasia patients with pyridoxine-responsive seizures in the literature, so early diagnosis of infantile hypophosphatasia in the clinically compatible patients allows more effective postnatal care/management and genetic counseling for further pregnancies.

Keywords: Infantile hypophosphatasia, *ALPL* gene, novel mutation, pyridoxine responsive

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

There is a limited number of hypophosphatasia patients with pyridoxine-responsive seizures in the literature.

WHAT THIS STUDY ADDS?

We reported the case of an infantile hypophosphatasia patient who presented with pyridoxine-responsive seizures. A novel homozygous mutation in the *ALPL* gene was detected.

Introduction

Hypophosphatasia (HPP) [Online Mendelian Inheritance in Man (OMIM) 146300, 241500 and 241510] is a hereditary congenital bone disease marked by a deficiency of alkaline phosphatase (ALP) activity in the liver, bones, and kidneys and is associated with defective skeletal mineralization. It was

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This study was presented as a poster presentation in the 1st Ege Endocrinologic Diseases and Genetic Symposium and was published as a poster presentation in the special edition 'An Infantile Hypophosphatasia Patient with a Homozygous Mutation in the ALPS Gene. J Clin Res Pediatr Endocrinol 2015;7(Suppl 2):79'

first described in 1948 by Rathbun (1). HPP has diverse phenotypes and is classified into seven subtypes based on the age of onset and clinical features: perinatal, benign prenatal, infantile, childhood and adult type, odontohypophosphatasia, and pseudohypophosphatasia (2). There are several patterns of inheritance. As already stated, inheritance can be autosomal dominant or recessive, but *de novo* mutations have also been described (3). Severe forms of HPP, primarily perinatal and infantile, are inherited in an autosomal recessive manner. Moderate forms (mHPP), primarily prenatal benign, childhood, adult, and odontohypophosphatasia, mostly result from heterozygosity for dominant severe alleles or from compound heterozygosity for severe and moderate alleles, and more rarely from two moderate alleles. The prevalence of severe HPP was estimated at 1/300,000, and prevalence of dominant mHPP was estimated to be 1/6370 in the European population (4).

HPP is caused by loss-of-function mutations in the *ALPL* gene, the gene encoding the isoenzyme tissue-nonspecific ALP (TNSALP), which is located in the chromosome region 1p36.1-1p34. It consists of 12 exons distributed over 50 kb. Many mutations have been described on the *TNSALP* gene, mostly in European, North American, and Japanese patients (5).

The clinical severity is associated with the age at diagnosis and the lack of TNSALP activity, except for odontohypophosphatasia where only the teeth are affected. Patients with the perinatal form of HPP almost always die around birth due to impaired development of the lungs and the severe hypomineralization of their bones (6). Infantile type symptoms are similar to, but typically less severe than, perinatal form and are recognized before 6 months of age. Childhood- and adult-onset HPP typically present with premature loss of deciduous teeth, rachitic changes in children and osteopenia, recurrent fractures, and pseudofractures with early loss of adult dentition, common osteomalacia in adult, and delayed healing of fractures (7). In this manuscript, we present an infantile patient diagnosed with HPP who had a novel homozygous mutation in the *ALPL* gene.

Case Report

A one-month-old female infant was evaluated because of limb shortening, hypercalcemia, and epilepsy. She was born at the 39th week of gestation by Caesarean section with a birthweight of 2070 g as the first child of consanguineous parents. Her prenatal ultrasound and family medical history were normal. The APGAR score was 7 at 1 minute and 10 at 5 minutes after birth. Metabolic acidosis and seizures were observed in the first day of life. Intubation was attempted because of respiratory failure. She was referred to our hospital because of myoclonic seizures that were not controlled after phenobarbital and phenytoin therapy. On her physical examination at one month old, her weight was 2470 g (<3th centile), her height was 50.5 cm (3th-10th centile), and her head circumference was 35.5 cm (10th-50th centile). A flattened facial appearance, broad forehead, flattened nasal bridge, bilateral low-set ears, short neck, narrow thorax, shortening of left arm and dimples on knees, hepatomegaly palpable 2 cm below the right costal margin, and 2/6 systolic murmur were detected (Figure 1).

Her skull and long bones radiographies showed distorted trabeculation, reduced mineralization, metaphyseal irregularities, cupping, diaphyseal shortening, shortness of the right humerus, ulna, and radius (Figure 2). Craniosynostosis was not observed. Her echocardiography detected ostium secundum atrial septal defect. Renal ultrasonography revealed medullary hyperechogenicity suggesting medullary nephrocalcinosis. Her ophthalmologic examination, cranial magnetic resonance imaging, and electroencephalography were normal.

The results of blood serum tests were as follows: calcium 14.5 mg/dL (N: 8.8-10.2), phosphorus 7.1 mg/dL (N: 4.5-5.5), ALP 1 IU/L (N<70), magnesium 1.8 mg/dL (N: 1.7-2.55), parathyroid hormone (PTH) 5.5 pg/mL (N: 15-65), and 25-hydroxy vitamin D3 49.5 ng/mL (the cut-off for vitamin D_3 deficiency is <20). Urinary calcium excretion was high with a calcium/creatinine ratio of 0.64. Her complete blood count and routine biochemistry tests for renal, liver, thyroid functions, immunoglobulins, tandem mass spectrometry, urinary and plasma amino acid analysis, urinary organic acid analysis were all found to be within normal limits.

Figure 1. Facial phenotype of the patient showing flattened facial appearance, broad forehead, flattened nasal bridge, bilateral low-set ears, short neck, and narrow thorax

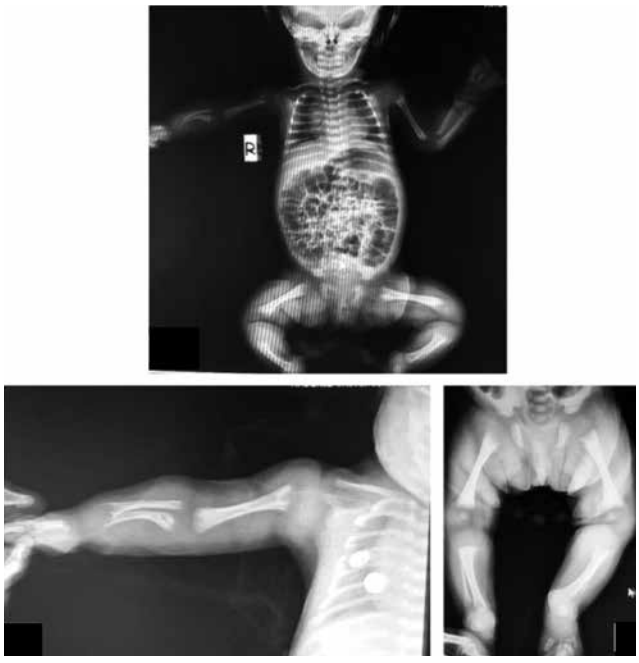

Figure 2. Radiographic imaging of the patient showing distorted trabeculation, reduced mineralization, metaphyseal irregularities, cupping, diaphyseal shortening, shortness of the right humerus, ulna, and radius

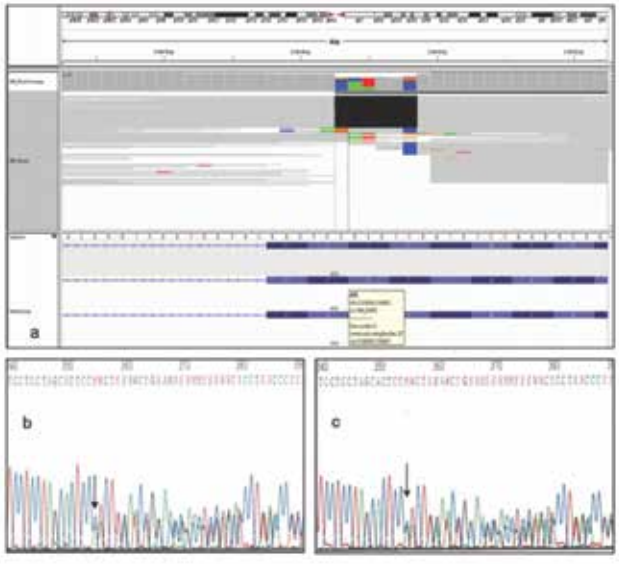

Figure 3. Diagram showing homozygous deletion (c.799_840delCACTTC) on *ALPL* gene of the proband (a). Electropherograms of father (b) and mother (c) confirming heterozygous presence of the same mutation that was detected in the proband

Her seizures, refractory to previous antiepileptic therapy, were under control after pyridoxine (vitamin B6) administration (100 mg/day initial, 50 mg/day maintenance dose, PO). Intravenous hydration with saline plus furosemide was started at the time of admission to treat hypercalcemia, and dietary calcium intake was restricted. This therapy had minimal effect on calcium levels, and serum calcium was 13.8 mg/dL on the 3rd hospital day. The hypercalcemia was resistant to other treatment options including prednisolone (2 mg/kg/d). Normocalcemia was achieved with a single dose of calcitonin (4 U/kg/d).

Chromosome analysis of peripheral leukocytes using high-resolution binding technique showed a normal 46,XX karyotype. A homozygous p.267_268delHF (c.799_804delCACTTC) mutation was detected in *ALPL* gene. The parents were also heterozygous for the new mutation. Genomic DNA of both parents and the index patient was screened for mutations in *ALPL* gene, and Sanger-sequencing revealed a novel homozygous mutation, p.267_268delHF (c.799_804delCACTTC), in the index patient (Figures 3a, 3b, 3c). This alteration was not annotated in National Center for Biotechnology Information or the human gene mutation database, and both parents were heterozygous carriers for this novel mutation in *ALPL* gene.

Discussion

The clinical presentation of HPP is variable and is thought to reflect the severity of the mutation in the *ALPL* gene as well as the mode of inheritance (dominant vs. recessive). The clinical characteristics of infantile type of HPP are respiratory complications, premature craniosynostosis, demineralization, rachitic changes in the metaphyses, hypercalcemia, short stature, and premature loss of primary teeth (8). Clinical features, age, bone mineralization, elevated serum concentrations of calcium and phosphorus, and low serum ALP enzyme activity helps differentiate HPP from other conditions. Differential diagnosis of infantile HPP includes osteogenesis imperfecta type 2, thanatophoric dysplasia, campomelic dysplasia, chondrodysplasia with bone mineralization defects. In addition, irritability, poor feeding, failure to thrive, hypotonia, and seizures place the infantile type in a broad differential diagnosis that includes inborn errors of energy metabolism, organic acidemia, and non-accidental trauma.

HPP is a distinct variant of rickets or osteomalacia and due to the block of mineral uptake into the skeleton, hypercalcemia and hyperphosphatemia are common in infantile HPP. Low serum PTH levels, normal serum 25-hydroxy vitamin D and 1,25-dihydroxy vitamin D, and hypercalciuria are the other typical biochemical findings of the disease (9). Radiological findings may be similar to rickets. It can be distinguished from rickets by low ALP levels (10). In the presented patient, HPP was considered with onset before 6 months of age, phenotypic appearance, skeletal deformities, hypercalcemia, remarkably low level of ALP, and low PTH level. The phenotypes, extremities, as well as calcium and ALP levels were normal in both parents.

We also observed seizures refractory to antiepileptic therapy but responsive to pyridoxine. Only a few HPP patients were reported in the literature with pyridoxine-responsive

seizures, and it was considered to be an indicator of disease severity. Several specific mutations have been suggested to be responsible. The mechanism of pyridoxine-responsive seizures in HPP is explained by defective metabolism of pyridoxal 5-phosphate (PLP), which is the phosphorylated form of pyridoxine. PLP, one of the natural substrates of ALP, is the active compound by which pyridoxine mediates essential enzyme activity; PLP deficiency in the central nervous system may reduce seizure threshold by reducing neurotransmitter (GABA) synthesis (11,12,13,14,15,16).

Molecular analysis of the *ALPL* gene can verify the genetic defect. A homozygous p.267_268delHF (c.799_804delCACTTC) mutation was detected in *ALPL* gene. To date, at least 210 distinct mutations and 12 polymorphisms have been reported. Most of the reported mutations (79.3%) are missense mutations, and the remaining reported mutations are deletions (small deletions 10.1%, large deletions 0.9%), splicing mutations (4.1%), nonsense mutations (2.8%), small insertions (1.8%), a complex deletion + insertion, a *de novo* mutation, and a nucleotide substitution affecting the major transcription initiation site (17). After searching the single nucleotide polymorphism database and the human gene mutation database, we found that p.267_268delHF (c.799_804delCACTTC) mutation is absent from the two databases; thus, our patient carry a novel missense mutation in the *ALPL* gene.

Although enzyme replacement therapy may provide a therapeutic option, still there is no current therapy for HPP. Recently, clinical trials have been undertaken using recombinant human TNSALP especially in a small number of infants and young children with severe HPP as well as in juveniles and adults, with promising results for bones, and pulmonary and physical functions (18). However, further investigation is needed in phenotype-genotype correlation studies to predict the severity of the disease, and clinical trials to evaluate treatment strategies for HPP. In our patient, when the health status stabilized, she was referred to another university hospital by air ambulance to receive enzyme replacement therapy.

In conclusion, infantile HPP has a high mortality rate, and it is important to consider the infantile HPP in the differential diagnosis of skeletal deformities, hypercalcemia, and low level of ALP. Molecular diagnosis is necessary to better understand the molecular basis of the disease, to improve the outcomes of genetic counseling, and may offer the possibility of future prenatal diagnosis.

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Ethics

Informed Consent: Informed consents were obtained from the patient parents.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Banu Güzel Nur, Design: Banu Güzel Nur, Ercan Mıhçı, Data Collection or Processing: Banu Güzel Nur, Gamze Çelmeli, Erdoğan Soyucen, İffet Bircan, Analysis or Interpretation: Banu Güzel Nur, Esra Manguoğlu, Literature Search: Banu Güzel Nur, Ercan Mıhçı, Writing: Banu Güzel Nur.

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Case Report

Pseudotumour Cerebri Presentation in a Child Under the Gonadotropin-Releasing Hormone Agonist Treatment

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ABSTRACT

Gonadotropin-releasing hormone analogues are common treatment option in central precocious puberty in childhood as well as in endometriosis, infertility, and prostate cancer in adults. Pseudotumor cerebri is a rare side effect observed in adults. We present the case of a girl with precocious puberty treated with triptorelin acetate who developed pseudotumor cerebri after the 4th dose. She had headaches, and her blood pressure was detected to be above the 99 percentile. There were no causes underlying of hypertension such as cardiac, renal, or endocrine. Neurological examination was normal except bilateral papilledema. Cranial magnetic resonance imaging was normal. Cerebrospinal fluid (CSF) opening pressure was elevated. Triptorelin therapy was ceased and acetazolamide was applied; CSF pressure returned to normal. We observed pseudotumor cerebri after precocious puberty treatment, a finding for the first time ever seen in childhood.

Keywords: Gonadotropin-releasing hormone agonist treatment, side effect, pseudotumor cerebri

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Gonadotropin-releasing hormone (GnRH) analogues are common treatment option in central precocious puberty. Sterile abscess at the injection site, menopause-like symptoms, headache, emotional disorders, syncope, osteoporosis, vasodilatation, and peripheral edema are rare side effects in children.

WHAT THIS STUDY ADDS?

We presented a pediatric patient who developed pseudotumor cerebri after the treatment of precocious puberty, the side effect for the first time ever seen in childhood. So, we suggest that complaints like headache, nausea, vomiting, and double vision in pediatric patients treated with GnRH analogue should consider the presence of pseudotumor cerebri.

Introduction

Gonadotropin-releasing hormone (GnRH) analogues are common treatment option in central precocious puberty. Synthetic leuprolide acetate and triptorelin acetate are also used safely in the treatment of endometriosis, infertility, and prostate cancer. Sterile abscess at the injection site, menopause-like symptoms, headache, emotional disorders, syncope, osteoporosis, vasodilatation, and peripheral edema are rare side effects in children (1,2). Bone pain, micturition problems, hypersensitivity (itching, skin rash, fever), gynecomastia, depression, easy and quick to anger, headache, nausea, muscle pain, joint pain, excessive sweating, fatigue,

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sleep disturbances, pain at the injection site, predisposition to hypertension, and thrombosis are principally observed in adults (3,4,5,6,7).

Here, we present the case of a girl with precocious puberty treated with triptorelin acetate who developed pseudotumor cerebri, a side effect for the first time ever seen in childhood.

Case Report

Nine-year-old girl was admitted because of breast development which had started 10 months before. Rapid height growth, adult body odor, and vaginal discharge are available. There was no history of drug use or chronic illness. On physical examination, weight was 34 kg [80 p, 0.8 standard deviation score (SDS)], height 138.3 cm (85 p, 1.06 SDS), arterial blood pressure 100/60 mmHg, thelarche bilateral Tanner stage 3, pubic hair stage 2, and axillary hair was not detected. Her body mass index was normal (17.7 kg/m^2, 0.5 SDS), and she did not have history of any recent onset of weight gain. Patient's bone age was 10.5 years. On pelvic ultrasonography (US), the length of the uterus (45 mm) and ovarian volumes (2.5 mL and 3.1 mL) were observed in pubertal size. Hypophysis magnetic resonance imaging (MRI) was normal. Gonadotropin levels were within pubertal range [follicle-stimulating hormone (FSH): 1.97 mIU/mL, luteinizing hormone: 1 mIU/mL]. Her bone age demonstrated fast progress of 1.5 years in 6 months, and annual follow-up height growth was 7.5 cm. Depot 3.75 mg of triptorelin acetate per month was started because of the early puberty. She had headaches after the 4^{th} dose, and her blood pressure was 130/80 mmHg (>99 percentile). The patient's previous medical records revealed no history of hypertension, and her blood pressure increased after treatment. Multiple measurements showed systolic/diastolic blood pressure in the range of 130-155/85-110 mmHg. Other system examinations were normal. Complete blood count, renal function tests, and serum electrolyte levels were in normal limits. Echocardiography analysis and renal Doppler US were normal. Plasma renin activity and aldosterone levels were observed in the normal range. There was no abnormality related to patient's neurological examination except bilateral papilledema. On cranial MRI, space-occupying mass was not observed and ventricular system was intact. The orbital section of MRI revealed bilateral optic nerve enlargement. Lumbar puncture was performed, and an elevated initial cerebrospinal fluid (CSF) opening pressure was detected (46 cm H_2O, normal range: 15-25 H_2O). Based on these findings, the patient was diagnosed with pseudotumor cerebri, and triptorelin therapy was ceased. Except GnRH analogue treatment, there were no any risk factors that might lead to pseudotumor cerebri such as obesity, renal failure, drugs, etc. The patient improved with treatment of acetazolamide, and the CSF pressure and fundoscopic examinations returned to normal. So, we think that the high blood pressure might be due to the increased intracranial pressure.

Discussion

We presented a patient who developed pseudotumor cerebri after treatment with a GnRH analogue, triptorelin acetate. GnRH analogues are in the form of injectable depot, administered 3.75 mg monthly or 11.25 mg every three months.

The common side effects of GnRH analogues in both adults and children are as follows: sweating, flushing, sleep disorders, psychiatric disorders such as depression, bone mineral density reduction with long-term use, and osteoporosis because of their menopause-like effects (1,3,4). Also, side effects such as headaches, muscle pain, allergy, skin eruption, sterile abscess, hypertension, hypercoagulability may occur (4,5,6). Cardiovascular effects reported in adults are associated with hypoestrogenism and hipoandrogenism (7,8). In reports, two patients with central precocious puberty who developed hypertension because of triptorelin acetate treatment are explained by the same mechanism (8,9).

Our patient was diagnosed with central precocious puberty and treated with triptorelin acetate. Under the treatment, she developed headaches and hypertension. There were no causes underlying of hypertension such as cardiac, renal, or endocrine. Neurological examination was normal except bilateral papilledema. Cranial MRI was normal. On lumbar puncture, initial CSF opening pressure was elevated. The patient was diagnosed with pseudotumor cerebri based on those findings, and triptorelin therapy was ceased. We could not find a reason to explain intracranial hypertension such as Cushing disease, hypoparathyroidism, iron deficiency anemia, obesity, or any history of drug use. The patient improved with treatment of acetazolamide, and the CSF pressure and fundoscopic examination returned to normal.

Pseudotumor cerebri is characterized by a normal neurological examination except for sixth cranial nerve paralysis and papilledema without pathological findings on brain MRI (10). While there is no reason in the majority of adult patients, an underlying cause is determined in 53-77% of pediatric cases. The annual incidence is 1/100.000 (11). Endocrine abnormalities, metabolic problems, infections, trauma, medications, and venous sinus thrombosis constitute the etiology. Among the most common causes of pseudotumor cerebri due to drugs, oral contraceptives, cyclosporine, isotretinoin, phenytoin, and steroids may be considered. The symptoms in children were headache, nausea, vomiting, blurred vision, diplopia, neck stiffness, photophobia, and retro-orbital pain. Prognosis is better in children than adults. Lumbar puncture can be reduced to the CSF pressure within normal limits and provides therapeutic effects on symptoms in most children (12). Lowering of the intracranial pressure as soon as possible is crucial in order to prevent compression of the optic nerves and vision loss. Therapeutic options of pseudotumor cerebri include medical and surgical modalities, but causal factors such as drugs and

obesity should be eliminated primarily. Medical management in childhood is essential. The first-line treatment is acetazolamide. Pseudotumor cerebri has a good prognosis with medical therapy in the pediatric population (13).

In the literature, it was reported that only four adult patients developed pseudotumor cerebri after the use of GnRH analogue (14,15,16,17). One of the patients used triptorelin acetate and the others used leuprolide acetate. All patients had different disorders; however, before the GnRH analogue treatment, none of them had neither predispositions such as obesity, Addison disease, drugs etc. nor clinical findings consistent with pseudotumor cerebri. After stopping the GnRH analogue treatment and starting the convenient treatment, all patients returned to normal. The authors did not find any other reasons, thus, those clinical events were accepted as a side effect of the GNRH analogue treatment (14,15,16,17).

Pseudotumor cerebri development mechanism after GnRH treatment is not fully understood. Possible mechanism is: GnRH analogue causes a short-term increase of sex steroids after injection and a mild venous sinus thrombosis develops due to high serum levels of steroids. Also they may cause non-obstructive thrombosis of the dural venous sinuses by creating a venous hypertensive state and prevent the CSF drainage. Therefore, benign intracranial hypertension (BIH) arises (14). As well as in our case, after cessation of treatment, normalization of CSF pressure and eye examination suggests strongly the role of GnRH analogues in the BIH etiology. Complaints such as headache, nausea, vomiting, and double vision in pediatric patients treated with GnRH analogue should consider the presence of pseudotumor cerebri and fundus examination should be performed.

We presented a pediatric patient who developed pseudotumor cerebri after treatment of precocious puberty. To our knowledge, this side effect of triptorelin acetate is for the first time ever seen in childhood.

Ethics

Informed Consent: It was taken.
Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Ülkü Gül, Ayşe Kaçar Bayram, Mustafa Kendirci, Nihal Hatipoğlu, Deniz Okdemir, Hakan Gümüş, Selim Kurtoğlu, Design: Ülkü Gül, Ayşe Kaçar Bayram, Mustafa Kendirci, Nihal Hatipoğlu, Deniz Okdemir, Hakan Gümüş, Selim Kurtoğlu, Data Collection or Processing: Ülkü Gül, Ayşe Kaçar Bayram, Mustafa Kendirci, Nihal Hatipoğlu, Deniz Okdemir, Hakan Gümüş, Selim Kurtoğlu, Analysis or Interpretation: Ülkü Gül, Ayşe Kaçar Bayram, Mustafa Kendirci, Nihal Hatipoğlu, Deniz Okdemir, Hakan Gümüş, Selim Kurtoğlu, Literature Search: Ülkü Gül, Ayşe Kaçar Bayram, Mustafa Kendirci, Nihal Hatipoğlu, Deniz Okdemir, Hakan Gümüş, Selim Kurtoğlu, Writing: Ülkü Gül, Ayşe Kaçar Bayram, Mustafa Kendirci, Nihal Hatipoğlu, Deniz Okdemir, Hakan Gümüş, Selim Kurtoğlu.

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Brief Report

Attitudes of Pediatricians Regarding Prevention and Treatment of Vitamin D Deficiency

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ABSTRACT

To determine the adherence of pediatricians to the nationwide 'Vitamin D Prophylaxis Program' and to evaluate their attitudes about vitamin D intake. The study was conducted using the Turkish National Pediatrics Association network. The pediatricians were asked to respond to an online questionnaire that included five questions on 'What dose of vitamin D they recommend for supplementation?', 'At what age they start vitamin D supplementation?', 'Supplementation method', 'Clichés and truths about vitamin D', and 'High-dose vitamin D therapy indications'. Responses of 167 pediatricians were evaluated in this study. 75.5% of pediatricians indicated that they recommended vitamin D supplementation in a daily dose of 400 IU. 47.1% started vitamin D supplementation by the end of the 2nd week. 7.83% of pediatricians suggested doubling the daily dose of vitamin D supplementation in infants with delayed tooth eruption, 19.9% suggested immediate cessation of vitamin D supplementation in infants with small anterior fontanels. This study showed that the majority of the pediatricians still prescribe vitamin D prophylaxis late, recommend high doses of vitamin D in cases of delayed tooth eruption, and think that low serum 25-hydroxy vitamin D level regardless of alkaline or phosphatase parathyroid hormone measurement is an indication for high-dose vitamin D (stoss) therapy. These results suggest a need for new training programs focusing on vitamin D supplementation.

Keywords: Vitamin D deficiency, prevention, pediatrician attitudes

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Introduction

While there is an increased awareness on the critical role of vitamin D on health, vitamin D deficiency is still an important health problem due to the influence of social, cultural, and geographic factors (1). Symptomatic vitamin D deficiency leads to morbidities including congenital and infantile rickets, hypocalcemic

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convulsions, dilated cardiomyopathy, skeletal myopathy, and osteomalacia from fetal life to adulthood. It may even lead to death. All these conditions are reversible and also preventable. Therefore, it is essential to clarify the responsibilities of families, physicians, and healthcare system in preventing vitamin D deficiency and to establish vitamin D supplementation as a public health priority similar to vaccination (2).

A nationwide vitamin D prophylaxis program that included free distribution of vitamin D drops to all newborns and infants (0-12 months) attending primary health centers throughout the country was initiated in 2005 in Turkey (3). However, it has been reported that physician attitude for vitamin D supplementation is not always in compliance with guidelines and also that some parents are unwilling to keep up with recommendations (4,5,6).

This study aimed to determine the adherence of pediatricians to the guidelines of the program and to evaluate their fundamental attitudes about vitamin D deficiency and vitamin D supplementation.

Methods

The study was conducted making use of the Turkish National Pediatrics Association Network. The questionnaire was sent to 1800 pediatricians. The pediatricians filled out an online questionnaire regarding their policy on vitamin D supplementation.

The questionnaire consisted of 5 questions. The first part three questions were on vitamin D supplementation practices:

What daily dose of vitamin D supplementation do you recommend? 1200 IU, 400 IU, 600 IU, 800 IU.

At what age do you recommend to start vitamin supplementation? Soon after birth, at the end of the 1st week, at the end of the 2nd week, at the end of the 3rd week, after age one month.

Which method do you prefer for vitamin D supplementation? Three drops orally once daily (400 IU/day), eight drops orally once daily (1000 IU/day), one vial (300.000 IU) orally once a month, one vial (300.000 IU) orally every two months, one vial (300.000 IU) intramuscularly every two months.

We also asked whether their practices were influenced by some common non-scientific beliefs.

Clichés and truths about vitamin D intake (giving double dose of daily vitamin D prophylaxis in infants with delayed tooth eruption, abstaining from vitamin D supplementation in infants with small anterior fontanelles, giving extra doses of vitamin D to infants with delayed walking and/or leg bowing).

The last question queried whether they followed the under-mentioned indications for high-dose vitamin D therapy (stoss therapy) in their clinical practice.

High-dose vitamin D therapy indications for all newborns and infants consist of a 25-hydroxy vitamin D [25(OH)D] level <15 ng/mL, an elevated serum alkaline phosphatase levels in addition to a serum 25(OH)D level of <15 ng/mL, and presence of craniotabes in addition to a serum 25(OH)D level <15 ng/mL.

Results

A total of 167 pediatricians completed the questionnaire. 75.5% of them indicated that they routinely recommended daily vitamin D prophylaxis in a dose as 400 IU. 10.2% of respondents recommended vitamin D prophylaxis in a daily dose of 800 IU. The remainder of the responders stated they recommended daily doses of 1200 IU and 600 IU. 28.7 % of respondents recommended beginning vitamin D prophylaxis soon after birth and 47.3% recommended beginning by the end of the 2nd week. 86.2% of respondents recommended daily 3 drops for vitamin D prophylaxis, while 11.38 % recommended daily 8 drops and 1.2% administration of oral 300.000 IU every 2 months.

7.8% of respondents suggested doubling the daily dose of vitamin D prophylaxis in infants with delayed tooth eruption, 19.9% suggested immediate cessation of vitamin D in infants with small anterior fontanelle, while 2.4% suggested giving 300.000 IU of vitamin D every two months to infants with delayed walking and 16.9% suggested giving 300.000 IU of vitamin D to infants with leg bowing (Table 1).

Discussion

The nationwide vitamin D prophylaxis program of the Turkish Ministry of Health successfully reduced the prevalence of rickets in Turkey from 6% in 1998 to 0.1% in 2008 in children under 3 years of age (3). This program is closely controlled by the Turkish Ministry of Health using a 'performance assessment system' that monitors primary care doctors' practices and rewards those who are compliant with the nationwide vitamin D prophylaxis program. In this program, the recommended daily vitamin D dose is 400 IU, which is compatible with the "Institute of Medicine (IOM) Committee's http://www.nationalacademies.org/hmd/ 2011 Report on Dietary Reference Intakes for Calcium and Vitamin D". This dose was chosen also because a daily 400 U supplementation of vitamin D was shown to be adequate to provide a serum 25(OH)D level >15

Table 1. For which of the following groups of infants is high-dose vitamin D therapy most appropriate?

Options	Percentage of responders (%)
Newborns and infants with serum 25-hyroxy vitamin D levels <15 ng/mL	34.73
Newborns and infants with serum 25-hyroxy vitamin D levels <15 ng/mL and elevated serum alkaline phosphatase levels	34.73
Newborns and infants with serum 25-hyroxy vitamin D levels <15 ng/mL and also craniotabes	30.54

ng/mL in >90% of all infants (7,8). In a large scaled survey study by the Ministry of Health to evaluate the success of the nationwide program, it was shown that of 2504 infants aged between 6 and 17 months, serum 25(OH)D level was >15 ng/mL in 73.6% (9). This is consistent with our survey findings that a similar percentage of pediatricians adhere to recommended vitamin D supplementation of the nationwide program. The survey also showed that a great majority of pediatricians preferred giving vitamin D supplementation daily and orally.

While this was encouraging, more than half of the pediatricians who responded to our questionnaire recommend to start supplementation later than the age recommended by the Ministry of Health. We believe this finding is mostly due to the misconception among pediatricians that the maternal transfer of vitamin D in utero would protect the infants against vitamin D deficiency in the first 3 weeks of life (10). However, because of the high incidence of maternal vitamin D deficiency, the American Academy of Pediatrics determined the beginning time of vitamin D supplementation as the first few days of life (6,11,12). We believe that a new information campaign is needed to change the physicians' attitudes for the starting age of vitamin D prophylaxis.

The results of our study reveal that non-scientific beliefs may influence clinical practice. 20% of the physicians thought that vitamin D supplementation should be stopped in babies with small anterior fontanels. We suppose that this belief is because of a misconception that if vitamin D deficiency can delay anterior fontanel closure, an early closure of fontanel should indicate vitamin D excess. Another stereotyped approach is erroneously considering bowing in infants automatically as a sign of vitamin D deficiency and prescribing high doses of vitamin D without additional findings though it may be due to other causes (physiological, skeletal dysplasia, or hypophosphatemic rickets). Similarly, 10% of pediatricians have stated that high-dose vitamin D treatment will accelerate tooth eruption and walking. This is consistent with a common belief among the parents.

Another interesting finding is that most pediatricians routinely analyze serum 25(OH)D and prescribe high doses of vitamin D (stoss therapy) without assessing the clinical symptoms and/or other biochemical and radiological parameters. We believe this attitude is due to the widely publicized extraskeletal effects of vitamin D (13). However, recently, treatment of vitamin D deficiency rickets has been revised and "stoss" or "single-dose" therapy dosage reduced to 50 000 units for the first year of life (14). It is also important to note that in Turkey, 300.000 IU vitamin D vials are available without prescription. The pediatricians' attitude as well as the common belief among the parents that high-dose vitamin D should be given to those children with delayed teething and/or walking, have led to a possible increase in Turkey of cases of vitamin D intoxication (15,16). In order to prevent vitamin D intoxication, the pediatricians need to be educated on this matter and over-the-counter sale of high-dose vitamin D preparations should be forbidden.

In conclusion, despite significant progress in the prevention and treatment of vitamin D deficiency in Turkey, some pediatricians still have incorrect attitudes such as starting vitamin D supplementation late, using high doses of vitamin D without a real indication, and accepting low serum 25(OH) D levels sufficient to begin high-dose vitamin D therapy. We believe there is a need for a new reinforcing education program among the pediatricians.

Ethics

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Şükrü Hatun, Design: Gülcan Seymen Karabulut, Şükrü Hatun, Data Collection or Processing: Enver Hasanoğlu, Analysis or Interpretation: Şükrü Hatun, Aysun Bideci, Literature Search: Şükrü Hatun, Writing: Gülcan Seymen Karabulut, Şükrü Hatun.

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Are Vitamin D Drops Containing 400 IU Daily Adequate for Preventing Vitamin D Deficiency?

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Dear Editor,

The adequacy of vitamin D intake is necessary to optimize the bone health during the rapid growing phase of infancy. Vitamin D also affects many organs playing an important role in maintaining general health. Vitamin D deficiency may be associated with cardiovascular disease, diabetes, cancer, and autoimmune diseases (1).

The incidence of vitamin D insufficiency and nutritional rickets has decreased in Turkey following the nationwide vitamin D prophylaxis programme undertaken by the Turkish Ministry of Health in 2005. This programme provides free vitamin D drops containing 400 IU daily for all children under 12 months of age (2). Our study demonstrated that this dose is not adequate for preventing vitamin D deficiency and insufficiency (3). Although İzmir has an abundance of sunshine almost throughout the year, our study showed that 40.9% of infants were sufficient, 28.4% of infants were insufficient, and 30.7% of infants were deficient in vitamin D levels on 400 IU of vitamin D supplementation (2). Halicioglu et al (4) also found that the rates of vitamin D deficiency and insufficiency were high in infants from a temperate region of Turkey who received daily 400 IU vitamin D supplementation. Because we found a high prevalence of vitamin D insufficiency and deficiency in infants who received 400 IU of vitamin D supplementation, we speculated that vitamin D prophylaxis dose should be increased from 400 IU to 600 or 800 IU in infants aged 0-12 months. We observed no patients with signs of hypocalcaemia, fits or tetany, and rickets in our study because dietary calcium intake was adequate in our patients despite vitamin D deficiency or insufficiency. We also reported high rates of maternal vitamin D deficiency and insufficiency in our study (2). The infants are at a high risk of vitamin D deficiency in the first year of life. Vitamin D supplementation is also important for decreasing the prevalence of severe early childhood caries with maintaining normal serum 25-hydroxy vitamin D (5).

Vitamin D prophylaxis dose might spark a debate in infants for maintaining general health. Further investigations would therefore be needed to clarify the optimal amount of vitamin D supplementation to the infants aged 0-12 months.

Keywords: Vitamin D insufficiency, vitamin D deficiency, vitamin D supplementation

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Crouzonodermoskeletal Syndrome with Hypoplasia of Corpus Callosum and Inferior Vermis

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Dear Editor,

Crouzonodermoskeletal syndrome) [Online Mendelian Inheritance in Man (OMIM) ID no. 612247] or Crouzon syndrome with acanthosis nigricans (CSAN) is a clinically and genetically distinct entity from the classic Crouzon syndrome (1). While classic Crouzon syndrome is caused by mutation in the *FGFR3* gene, CSAN is caused by only mutation p.A391E in the *FGFR3* gene. Other *FGFR3* gene mutations are not responsible of this syndrome, and they usually lead to hypochondroplasia or achondroplasia but not CSAN (2,3). Clinically, in addition to classic Crouzon syndrome, patients with CSAN have acanthosis nigricans and skeletal abnormalities (1,4). The phenotypic features include wide-set bulging eyes and underdeveloped upper jaw, craniosynostosis, midface hypoplasia, hypertelorism, proptosis, posteriorly rotated ears, and in some cases, hearing loss. Patients with CSAN often present with choanal atresia and hydrocephalus (4).

A ten-month-old girl presented with a facial dysmorphia at birth. There was no consanguinity between her parents. She was born at term weighing 3380 g with no perinatal complications. She had obstructive dyspnea at day 1 due to bilateral choanal atresia for which she was operated at day 9. At 9 months of age, she had coronal craniectomy surgery because of craniosynostosis due to bilateral coronal stenosis.

At presentation, her height was 68.7 cm [-1.13 standard deviation score (SDS)] and weight was 7.3 kg (-1.97 SDS). She had atypical facial features (midface hypoplasia, hypertelorism, craniosynostosis, brachycephaly, maxillary hypoplasia, exophthalmos, bilateral distinctive and low-set ears), lateral nystagmus on the bilateral eyes, and widespread acanthosis nigricans on all of curve regions as neck, bilateral axillae (Figure 1). Cranial magnetic resonance imaging (MRI) revealed hydrocephalus, hypoplasia of corpus callosum and inferior vermis (Figure 2). There was no pathology at abdominal ultrasonography and echocardiography. The audiogram did not reveal any pathology. The cognitive and motor development were delayed.

We detected a *de novo* heterozygous A391E (c.1172C>A) mutation in *FGFR3* gene in our patient. This syndrome is inherited in an autosomal dominant type although most cases are sporadic mutations (5). We detected the mutation in the patient but not in her parents and sisters (Figure 3). Therefore, our patient is a sporadic form of CSAN.

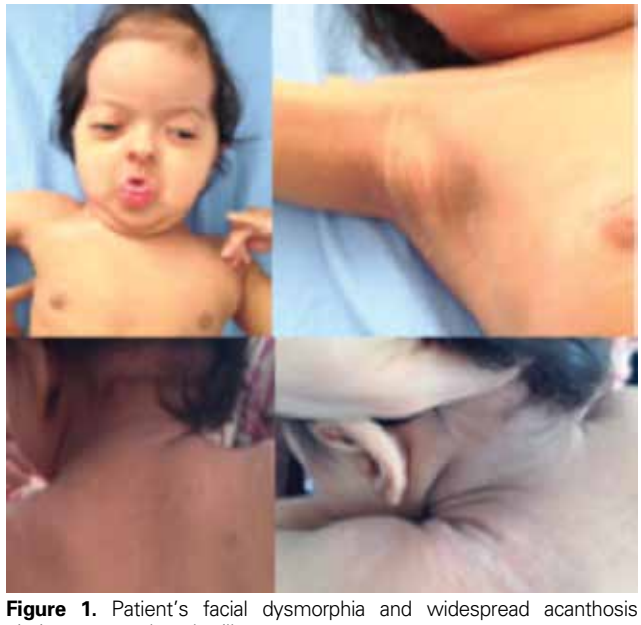

Figure 1. Patient's facial dysmorphia and widespread acanthosis nigricans on neck and axillae

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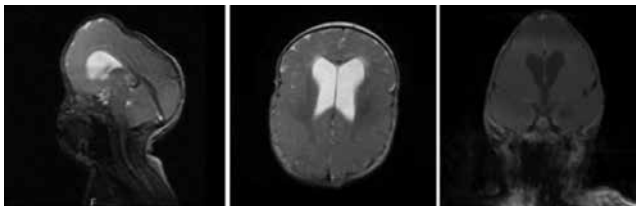

Figure 2. Patient's magnetic resonance imaging: presence of hydrocephalus and hypoplasia of corpus callosum and inferior vermis

Figure 3. Sight of the patient's pedigree and sequence (c.1172C>A)

As with other disorders caused by *FGFR* gene mutations, increased paternal age seems to be a risk factor (1). Our patient's father age was 42 years old.

To our knowledge, this is the first case of CSAN with hypoplasia of corpus callosum and inferior vermis. This association may be coincidental. These patients should be investigated for other possible cranial MRI findings.

Keywords: Crouzonodermoskeletal syndrome, craniosynostosis, acanthosis nigricans, hypoplasia of corpus callosum, inferior vermis

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Ethics

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Concept: Fatih Gürbüz, Ali Kemal Topaloğlu, Bilgin Yüksel, Design: Fatih Gürbüz, Ali Kemal Topaloğlu, Bilgin Yüksel, Data Collection or Processing: Fatih Gürbüz, Ali Kemal Topaloğlu, Bilgin Yüksel, Analysis or Interpretation: Fatih Gürbüz, Serdar Ceylaner, Ali Kemal Topaloğlu, Bilgin Yüksel, Literature Search: Fatih Gürbüz, Ali Kemal Topaloğlu, Bilgin Yüksel, Writing: Fatih Gürbüz, Ali Kemal Topaloğlu, Bilgin Yüksel.

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