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"Cross-talk between endocrine system and other biological pathways: physiological and pathological implications in paediatric endocrinopathies"

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CHAPTER 1

General introduction and aims

Endocrine system exerts relevant effects on multiple organs and tissues. Pleiotropic and redundant functions of circulating hormones are due to the various receptors expressed on multiple target cells. Moreover, receptors of circulating hormones share common transducing elements with receptors of many other molecules. Consequently, endocrine system participate to an integrated network of mediators that communicate and coordinate responsive cells to achive effective functions in an appropriate fashion. The presence of such a complex interplay contribute to unravel previously unappreciated functions of circulating hormones and the mechanisms of coordination and integration of several pathways.

An example of this interplay is the complex interaction between endocrine and immune system. Cytokines and growth factors, in fact, after their binding to cell-surface receptos, activate common intracytoplasmatic signaling molecules. In the recent years the description of complex phenotypes, in which immunodeficiency and growth failure were associated at a different extent, greatly contributed to define how several signaling molecules play a role in both systems. As well as immunodeficiency, autoimmune diseases represent a unique model to study interactions between endocrine and immune systems.

Cardiovascular system also represents a biological system higly sensitive to the effects of circulating hormones as suggested by the presence of several cardiovascular and metabolic alterations found in many endocrine diseases. In fact, patient with growth hormone deficiency as well as thyroid dysfunction, may present an increased morbidity due to cardiovascular events and an increased incidence of atherosclerosis.

Aim of this project was to evaluate physiological and pathological implications of these complex molecular interactions on paediatric endocrinopathies starting from complex phenotype involving endocrine and other biological systems.

To this aim, we started from the study of 3 model of complex diseases:

- The model of autoimmune diseases

- The model of Growth Hormone (GH) Deficiency (GHD)
- The model of Congenital Hypothyroidism (CH)

  Moreover, during the study of patients affected with Growth Hormone and thyroid disorders we also identified new genetic causes of GH deficiency and hyperthyroidism, highlithing new insights in the genetics of endocrine diseases, as described in the last chapter of the thesis.

CHAPTER 2
THE MODEL OF AUTOIMMUNE DISORDERS AS COMPLEX DISORDERS INVOLVING BOTH IMMUNE AND ENDOCRINE DISEASE: GENETIC AND MOLECULAR BASIS

2.1 Introduction

Autoimmune diseases represent a significant health burden in the developed world affliciting 5-10% of the population (1), and a sizable percentage of these diseases involve an untoward immune response against an endocrine organ. Virtually any endocrine organ can be targeted by the immune system as part of an autoimmune response, and frequently responses to multiple organs can occur in the same patient as part of a polyglandular autoimmune syndrome. More common endocrine autoimmune syndromes include Hashimoto's thyroiditis, Graves' disease, and type 1 diabetes, whereas more rare syndromes include Addison's disease, oophoritis, lymphocytic hypophysitis, and hypoparathyroidism. For years, the etiology and pathogenesis of these disorders have remained obscure, but the diseases are generally thought to involve a cellular and humoral immune response. Autoimmunity is the result of combinational input from multiple genetic loci, breakdown of immunological tolerance mechanisms, either at a central and a peripheral level, and enviromental interactions.

2.2 Genetic Component of Autoimmune diseases

As for genetic component, it has been universally recognized for the past 30 years that most autoimmune diseases have a polygenic basis. There are findings that strongly support that common groups of genes may contribute to development of clinically distinct forms of autoimmune disease: first, most autoimmune disease are thought to arise from alterations in the immune system; second, co association of different autoimmune diseases is often found in families or individuals; third, analysis of genome-wide linkage results demonstrate that multiple autoimmune diseases share common susceptibility loci (2). Although association between the HLA specifities and autoimmune conditions has been established for a number of diseases, HLA represents

only a part of the genetic susceptibility factors, which “per se” is not able to explain the occurrence of the disease.

Recent progress has been made in identifying genetic polymorphism that are associated with risk of disease in the common autoimmune disorders. Many of these polymorphism highlight the central role of T cells in the breakdown of self-tolerance. Genetic risk for autoimmunity has been convincely demonstrated for genes expressed in T cells, such as those encoding protein tyrosine phsphatase non-receptor 22 (PTPN22) and cytotoxic T lymphocyte associated 4 (CTLA4). Like those associated with the HLA locus, the mechanisms by which polymorphism in these genes lead to disease risk also remain obscure. The lack of clear mechanism for these risk alleles is not surprising because genetic polymorphism in individual genes alone are often not sufficient to cause disease and reflect the fact that autoimmunity is the result of combinatorial input from multiple genetic loci and environmental interactions (3).

2.3 Defects in Immune mechanisms involved in autoimmune diseases

Central and peripheral mechanism maintaining T-cell tolerance

Many autoimmune diseases seem to result from a failure to maintain T-cell tolerance. There are several levels at which T-cells are prevented from uncontrolled self reactivity. The first of those involves thymic selection. Pre-T-cells undergo a stringent process of selection, during which 95% are eliminated. Thymocytes with excessive reactivity of self antigens, which are presented by endogenous MCH molecules and activate thymocytes through interactions with T-cell receptors (TCRs), die through apoptosis. Surviving thymocytes undergo an additional process of positive selection and emerge from the thymus as mature T cells. Most of these T cells are various types of effector T cell, generally either CD4+ or CD8+.

A second major population of T cells, regulatory-T cells (T reg cells), are also produced during thymic selection. T reg cells have a key role in controlling the self-reactivity of effector Tcells in the periphery, because thymic selection does not completely eliminate autoreactive T-cells. Treg cells can also develop de novo in the periphery from precursors that might not be directely derived from Treg cells that emerge from the thymus (4).

Tregs, actively suppress pathological and physiological immune response, thereby contributing to the mainteinance of immunological self-tolerance and immune

homeostasis. These cells constitutively express the interleukin (IL-)-2 receptor α-chain (CD25) and their development and function depend on the expression of the transcription factor forkhead box P3 (FOXP3) (5). The molecular basis of IL-2 dependence of T-regs has not yet been addressed. In conventional T cells the effects of Il-2 are elicited by at least two major signalling pathways: one leads to the activation of the serine/ threonine kinase, AKT, and up-regulation of antiapoptotic molecules such as Bcl-2 and Bcl-x , and is required for Tcells survivals; the other leads to the activation of STAT5 and is required for Tregs cell proliferation and differentiation: it may also stimulate the expression of antiapoptotic molecules (6).

Several T-cell accessory molecules such as CTLA-4 (CD152) and lymphocyte-activation gene 3 (LAG3), expressed by T-regs, and CD80 and CD86 costimulatory molecules expressed by APCs contribute to the suppressive mechanism (5).

Genetic defects in central tolerance mechanism

AIRE (AutoImmune REgulator) encodes a transcription factor that regulates the thymic expression of a variety of self antigens and is involved in the mechanism of central tolerance. In humans mutations of A IRE cause the rare Mendelian autosomal recessive disease disorder Autoimmune Polyendocrine Syndrome 1 (APS 1), in which autoimmunity is focused largely on endocrine organs. APS-I (or APECED) represents a unique model of monogenic autoimmune disease. Recently it has been shown that a remarkably large number of organ-specific self antigens are expressed in the thymus. The absence of this expression can result in an escape from thymic deletion and the release of self-reactive T cells into periphery. A large number of self-antigens are expressed at highly variable levels in the thymic epithelium, and this might be a common underlying risk factor for a variety of complex autoimmune diseases, given the quantity nature of the thymic selection process (4).

In addition to defects in antigen expression and presentation, if thymocytes are unable to respond adequately to signals delivered through the TCR they will not be properly selected (4). For example, in the mouse it has been shown that a mutation in the gene that encodes ZAP70, a key TCR signalling molecules, results in reduced negative selection and subsequent escape of self-reactive T cells into periphery. A comprehensive analysis of ZAP70 polymorphism and related T-cell signalling molecules has not yet been carried out for human autoimmune disease (4).

Genetic defects in Tregs cells function

Depletion of CD25+CD4+ Tregs that are naturally arising in the immune system produces autoimmune disease in otherwise normal animals, and their reconstitution prevent the disease (5).

A role for Treg cells in human autoimmunity is demonstrated by the rare IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome). Mutations of the FOXP-3 gene lead to the deficiency or malfunction of natural Tregs and, consequently, the development of a similar autoimmune and/or inflammatory disorder in mice and humans (5).

Genetic defects in T-cell-co-stimulatory

In addition the part played by Tregs cells, the activation of peripheral T cells is also controlled by an array of costimulatory molecules on the T-cell surface, which modulate activation of these cells through the TCR. CD28, the inducible T-cell co-stimulator (ICOS) and the cytotoxic T-Lymphocyte-associated protein 4 (CTLA-4) are members of the immunoglobulin superfamily that are expressed on T cells and bind homologus ligands on APCs. CD28 and ICOS provide positive signals whereas CTLA-4 is generally a negative regulator of T cell activation. However, although CTLA-4 is generally hinibitory, it seems to be activating for Tregs cells. CTLA-4 knockout is associated with florid lymphoproliferation. CTLA-4 polymorphisms have been associated with a variety of human autoimmune diseases. CTLA-4 seems to be a general susceptibility locus for autoimmunity (4).

Defects of Apoptosis in autoimmune diseases

In the immune system, apoptosis encounters the proliferation of lymphocytes to achive an homeostatic balance, which allows potent responses to pathogens but avoid autoimmunity. Fas (CD59) is a transmembrane molecule belonging to the tumor necrosis factor (TNF) receptor superfamily and interacts with the Fas ligand (FasL), a type II transmembrane molecule (7). The Fas receptor triggers lymphocyte apoptosis by recruiting fas-associated death –inducing signalling complex (8). Heterozygous

mutations in CD59, CD59 ligand or caspase-10 underlie most cases of autoimmune lymphoproliferative syndrome (ALPS), a human disorder characterized by defective lymphocyte apoptosis, splenomegaly, lymphoadenopathy and autoimmunity, with expansion of TCR α/β CD4-CD8- double negative (DN) T cells (9).

The role of JAK-STAT signaling pathways in cellular homeostasis and its consequence in autoimmune diseases' process

The JAKs and other tyrosine kinases activate STAT proteins upon phosphorylation of a single, critical tyrosine residue. Tyrosine phosphorylation leads to homo- or heterodimerization of STAT monomers, which results in the subcellular relocalization of the STAT signal to the nucleus where they bind specific DNA sequences in the promoters of target genes and modulate transcription. Some STATs are further modified by phosphorylation on a conserved serine residue, which is thought to regulate transcriptional activation and may serve to integrate signals generated by other intracellular molecules. Nuclear STAT proteins are subsequently dephosphorylated on their tyrosine residue, translocate back to the cytoplasm, and are targeted for degradation. STAT proteins are unique among signalling proteins in their ability to transmit signals from the cell surface to the nucleus and directly participate in the regulation of gene expression. STAT proteins are important in mediating the effects of numerous cytokines, polypeptide growth factors, hormones, and oncoproteins. By modulating the expression of target genes, STAT transcription factors regulate a broad range of biological processes, including cell growth, differentiation, survival, and development. Seven STAT genes have been identified (STATs 1, 2, 3, 4, 5A, 5B, and 6), and the corresponding gene products share a high degree of structural similarity. Each STAT protein has a DNA binding domain, a src-homology 2 (SH2) domain necessary for homo- or heterodimerization, and a conserved tyrosine residue which can be phosphorylated by Janus protein kinases (JAKs) and other tyrosine kinases. Some STAT proteins also contain a conserved serine residue, which, upon phosphorylation, enhances transcriptional activity. A number of recent studies have defined both pro-apoptotic and anti-apoptotic signalling pathways mediated by STAT transcription factors The studies implicating STAT proteins in proapoptotic signalling have largely focused on STAT1 in interferon (IFN)-mediated growth inhibition andapoptosis; however, several recent reports using other effectors of STAT signalling, such as cytokines and ischemia, have also contributed to our understanding of the role of

STATs in promoting apoptosis. Studies implicating STATs in antiapoptotic signalling have mostly focused on cytokine signalling via STATs 3 and 5 (10). All these evidences suggest how STATs family is involved in many mechanisms of apoptosis.

STAT1

Recently, Stephanou *et al.* (11) demonstrated that STAT1 induces an autocrine death pathway by inducing expression of the Fas and FasL genes leading to apoptosis in cardiac myocytes exposed to ischemia/reperfusion. Stress-induced apoptosis caused by wound healing also appears to use STAT1. Keratinocyte apoptosis in response to epithelial scrape was reduced in keratinocytes from STAT1-null mice compared to their wild-type counterparts These studies indicate a broader role for STAT1 in cell growth and survival.

In vitro studies first indicated a role for STAT1 in apoptosis, and more recent data from transgenic mice overexpressing fibroblast growth factor receptor 2 (FGF2) support a pro-apoptotic role for STAT1 *in vivo*. Recent studies indicate that STAT1 can promote apoptosis through the upregulation of apoptotic regulatory genes, such as caspases, cell death receptors and their ligands, and possibly p21waf1 and Bcl-xL. Most studies linking STAT1 to caspase gene expression have focused on caspases 1 and 11, which are important for both cytokine protein processing and induction of apoptosis. STAT1 can also regulate expression of caspases 3, 7, and 8, which execute pro-apoptotic events. Stark *et al.* have proposed that STAT1 promotes apoptosis through the constitutive regulation of caspase expression. Analysis of caspase family members revealed that caspase 1,2 and 3 mRNA levels were low in STAT-1 deficient cells. In addition STAT1 activation can also lead caspase induction and activation. Infact, IFN γ induced caspase induction may be direct or mediated by STAT1.

STAT1 is also required for upregulation of molecules that directely mediate the death process such Fas and Fas ligand (Fas-L). In response to IFN- γ. Ifn induced Fas expression was severely impaired in microglial cells from STAT1 deficient mice (10).

STAT3

Evidences supporting an anti-apoptotic role for STAT3 comes from studies demonstrating that constitutive activation of STAT3 protects cells from programmed cell death.

The mechanism by which STAT3 supports cell survival has been linked to the transcriptional regulation of apoptotic regulatory proteins . Several studies have reported that the inhibition of STAT3 activation results in the downregulation of Bcl-xL and an upregulation of the pro-apoptotic gene, Bax. STAT3 may also promote cell survival through upregulation of p21waf1, which, in addition to its role in inhibiting cell cycle progression, appears to have an anti-apoptotic function through its ability to inhibit CDK activity. STAT3 also downregulates Fas expression through cooperation with c-jun, effectively suppressing apoptosis in human melanoma cell lines. Thus, STAT3 can contribute to suppression of Fas transcription, effectively inhibit Fas-mediated apoptosis, and promote cell survival (10).

STAT5

Two highly related genes encode STAT5 proteins, STAT5a and STAT5b. While STAT5 is activated by many factors involved in growth regulation and dfifferentiation, including growth hormone, prolactin, erythropoietin, granulocyte macrophage colony-stimulating factors (GM-CSF), trombopoietin, Il2, -3, -5 and 6, recent evidence indicate a prominent role for STAT5 in promoting survival of differentiating progenitor hematopoietic cells. Studies in STAT5a-/-5b-/- mice have demonstrated that STAT5 mediates an antiapoptotic effect in fetal red cells progenitors by directing inducing expression of antiapoptotic genes such as Bcl-xl. STAT5 activation is associated with survival of some leukemic cells and likely contributes to their resistance to undergo apoptosis. Further evidence to support a role for STAT5 in anti-apoptotic pathways comes from studies in mammary epithelial cells. In contrast to STAT3, STAT5 promotes proliferation, differentiation and possibly anti-apoptosis during mammary tissue remodelling (10).

Recently has been highlighted the essential role of STAT5 for cell homeostasis and tolerance. Two studies have demonstrated this role for STAT5: in one study authors demonstrate that TREGS homeostasis is dependent on the activation of STAT5, demonstrating that STAT5 deficient mice show reduced number of TREGS and that transient activation of STAT5 in IL-2 deficient mice increases the numbers of CD4+CD25+ Tregs in the periphery. Another study (12) (J W Snow, J Immunol 03) described that a subset of STA5A/5B deficient mice exhibited autoimmune pathology very similar to mice lacking IL-2 or its receptor components, characterized by lymphocytic infiltration of multiple organs, including the bone marrow. Affected mice

exhibited a dramatic increase in the number of memory CD4+ and CD8+ T cells infiltrating the bone marrow. In addition, Treg cells from STA5A/5B deficient mice did demonstrate reduced survival in response to IL-2 in vivo. These findings provide definitive evidences that STAT5 is crucial for the mainteinance of tolerance in vivo. In addition these findings indicate that regulatory T cells require the activation of STAT5, most likely by the IL-2R, to mantain their own homeostasis in vivo (12).
All these evidences hilight the role of STATs proteins as possible determinants of part of the process of thed autoimmunity.

2.4 Enviromental factors and autoimmunity: Infections and autoimmune disease

Virus infections have been long associated with autoimmune diseases, whether it is multiple sclerosis, diabetes, or myocarditis. Three potential mechanisms for virus-induced autoimmune disease are invoked: molecular mimicry, bystander activation and persistent virus infection (13). Molecular mimicry represents a shared immunologic epitope with a microbe and the host. In viral system, viruses have been shown to cross-reactive with host self proteins (14).
Bystander activation as a mechanism leading to autoimmune disease has gained support through the use of experimental models mirroring some of the features of autoimmune disease. Virus infections lead to a significant activation of APCs such as dendritic cells.These activated APCs could potentially activate preprimed autoreactive T-cells, which can than initiate autoimmune disease. In addition to this mode of bystander activation of T-cells, virus-specific T-cells also might initiate bystander activation (13)
Persistent viral infections can lead to immune-mediated injury due to the costant presence of viral antigen driving the immune response.

2.5 Clustering of Autoimmune Disease (CAD)

Introduction

Even though distinct autoimmune disorders may be associated, only rare patients exhibit a clear clustering of different diseases based on a polyreactive autoimmune process. Familial clustering of autoimmune disease within families could be explained by shared genotypes, shared environmental exposures, or some combination of both.
The existence of a mendelian inheritance for Clustering of Autoimmune Disease (CAD) has been well documented in several syndromes such as Autoimmune-Polyendocrinopathy Candidiasis Ectodermal Distrophy (APECED),

Immunodysregulation Plyendocrinopathy Enteropathy X linked Syndrome (IPEX) AND Autoimmune Lymphoproliferative Syndrome (ALPS). These 3 distinct clinical entities, each caused by by a single gene defect, have been associated with multiple autoimmune disorders (15).

IPEX

IPEX is a rare X-linked disorder of immune regulation resulting in the expression of multiple autoimmune disease. Patients can develop type 1 diabetes, enteropathy, eczema, variable autoimmune phenomena and severe infections. Older patients may present sarcoidosis, arthritis, glomerulonephritis, ulcerative colithis and neuropathy. The gene identified as responsible for this disorder is the transcriptional factor FOXP-3. FOXP-3 is mainly expressed in CD4+CD25+ T regulatory cells. Murine models with depletion of this T-lymphocyte population spontaneously develop T-cell autoimmune disease. Although 13 mutations of this gene have been at present identified, no genotype-phenotype correlation has been described (16).

ALPS

Autoimmune lymphoproliferative syndrome is a disorder characterized by chronic, non-malignant lymphoproliferation and autoimmunity, most commonly involving cells of hematopoietic origin. ALPS is due to a failure of apoptotic mechanism that helps maintain normal lymphocytes homeostasis, with a subsequent accumulation of lymphoid mass along with the persistence of autoreactive cells. Apoptosis is a mechanism of cell death triggered by specialized membrane receptors. Fas, also known as CD59 or Apo-1 or TNFRSF6 belongs to this family of protein and is the most efficient inducer of apoptosis in lymphocytes. After ligand binding, three molecules of Fas assemble into complexes. Fas signaling occurs through the interaction of Fas Associated Death Domain (FADD), a cellular adaptor and, subsequently, with procaspases 8 and/or 10 in a death inducing signaling complex (DISC) (17). Several forms of ALPS have been identified, which mainly differ for the molecule of the Fas/FasL pathway which is altered.

CASPASE 8 DEFICIENCY

In the mechanism of apoptosis, the CD59 receptor triggers lymphocyte apoptosis by recruiting Fas-associated death domain (FADD), caspase-8 and caspase 10 protein into a death-inducing signalling complex. Mutations of caspase 8 in mice cause embryonic lethality. A human kindred with caspase 8 deficiency has been described in 2002 (18) manifesting defective lymphocyte apoptosis and homeostasis and, in addition, (unlike others affected by ALPS), defects in activation of T lymphocytes, B lymphocytes and natural killer cells, which leads to immunodeficiency.

APECED

Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal-Distrophy (APECED) is a rare autosomal recessive disease (OMIM 240300) which affects many tissues especially endocrine glands. APECED is caused by mutations in the AutoImmune REgulator gene (AIRE), which maps to 21q22.3 and encodes a 55-kDa protein that acts as a transcription regulator (19). Over APECED-related mutations of the AIRE gene have been described so far (20).

The diagnosis is primarily based on the presence of two out of the three most common clinical features: hypoparathyroidism, Addison's disease, and chronic mucocutaneous candidiasis (19). Further clinical or latent autoimmune endocrine diseases may be associated. They include hypergonadotropic hypogonadism, Type 1 diabetes mellitus, and autoimmune thyroid disease. Non-endocrine autoimmune disorders include vitiligo, alopecia, urticaria-like erythema, chronic atrophic gastritis with or without pernicious anemia, celiac disease, malabsorption, autoimmune hepatitis, rheumatic diseases. Finally, other clinical features such as cholelithiasis, ectodermal dystrophy, acquired asplenia, cancer of the mucosae, calcifications of basal ganglia and tympanic membranes may also occur (21).

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CHAPTER 3

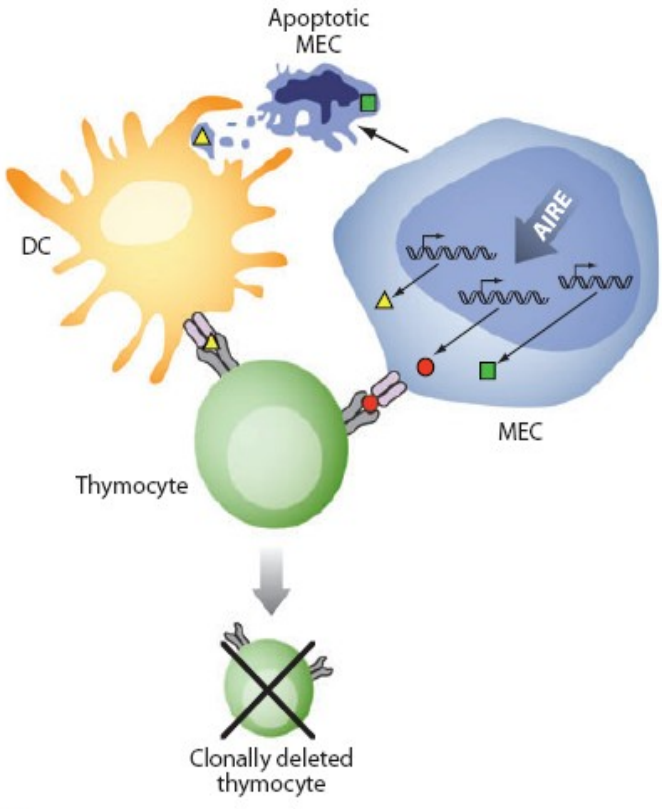
COMPLEX DISORDERS INVOLVING BOTH IMMUNE AND ENDOCRINE SYSTEMS: THE MODEL OF AUTOIMMUNE DISEASES CAUSED BY AIRE DEFICIENCY

3.1 Introduction and aims

Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal-Distrophy (APECED) is a rare autosomal recessive disease which affects many tissues, and especially endocrine glands(1).

APECED is caused by mutations in the AutoImmune Regulator gene (AIRE), which maps to 21q22.3 and encodes a 55-kDa protein (2,3). Recent studies document that this protein acts as a transcription regulator (4) and exerts a key role in the regulation of the central tolerance (5). Current view of Aire's major function in thymus is schematized in Fig. 1.

Fig. 1 Aire promotes clonal deletion of self-reactive thymocytes. Aire induces MEC expression of a broad repertoire of peripheral tissue antigens (PTAs), which are processed and then presented on surface-displayed MHC/HLA molecules. Soon after the induction of AIRE and PTAs, MECs die by apoptosis. Mature thymocyte percolate through the medulla, and, if their TCRs recognize an MHC:PTA complex in the appropriate affinity/avidity window, they will be overactivated and deletd from the repertoire. Thymocites can recognize MHC:PTA complex directly on MECs or indirectly on DCs that engulfed apoptotic MECs or MEC fragment. (5)



There is evidence that AIRE is mainly expressed by macrophages, and dendritic cells and is probably involved in the regulation of antigen presentation (6). So far, the exact role of AIRE is still unknown. Expression of AIRE by medullary thymic epithelial cells is subjected to tight regulation and requires the presence of thymocytes. In particular, it has been shown that expression of AIRE in mice is regulated by local production of lymphotoxin-alpha (LT-a) (7). Other TNF family members are involved in negative selection such as the newly discovered member cellular ligand for herpesvirus entry mediator and lymphotoxin receptor (LIGHT). LIGHT expression in transgenic mice induces apoptosis of CD4+/CD8+ double positive thymocytes suggesting that it is involved in the mechanisms of central tolerance.

In inherited disorders of immune system that are characterized by abnormal thymopoiesis such as Omenn syndrome and other severe combined immunodeficiencies, defects of thymocyte proliferation in response to T cell receptor (TCR) engagement may impair lymphotoxin expression and secondarily the mechanisms of central tolerance, thus leading to the autoimmune manifestations that are often observed in several primary immunodeficiencies. Furthermore, it has been recently demonstrated that thymic epithelial cells of patients affected with Omenn syndrome, a rare combined immune deficiency characterized by early onset of autoimmune manifestations, do not express AIRE protein (8).

The prevalence of APECED worldwide is very low. At least 58 APECED-related mutations of the AIRE gene have been described to date. In Italy the disease is also very rare but three hot spots areas have been identify. In Sardinia the typical AIRE mutation is characterized by a nonsense mutation on exon 3, defined as R139X. In Apulia a typical AIRE mutation is localized on exon 2 and is defined as W78R. In northern Italian populations R257X is very frequent and often associated with 1094-1106del113(10).

The diagnosis of APECED is primarily based on the presence of two of the three most common clinical features: hypoparathyroidism, Addison's disease, and chronic mucocutaneous candidiasis (1). Further clinical or latent autoimmune endocrine signs or diseases may be associated. APECED is characterized by the presence of organ and non-organ specific circulating autoantibodies. Even if the presence of certain

autoantibodies is often associated with specific signs or symptoms, their role in the pathogenesis of APECED is stillunknown.

Candidiasis is generally the first manifestation of the disease, usually appearing at around 5 yrs of age. It is often followed by hypoparathyroidism, before the age of 10 yrs, and later by adrenal insufficiency (1). However, the expression of the clinical phenotype is often partial in infancy. In fact, most of the patients with the classic triad of symptoms belong to the second or third decade of life. Clinical complications gradually appear over time, culminating in a complete clinical portrait of the disorder between the second and third decade of life. Correlation studies have so far failed to reveal a correlation between phenotype and genotype and, even among siblings with the same genotype, clinical phenotype can reveal wide heterogeneity.

Furthermore, recent reports highlight the possibility of unusual and peculiar components such as chronic respiratory disease and chronic inflammatory demyelinating neuropathy (CIDP) (11, 12). KCNRG , a putative potassium channel regulating protein, preferentially expressed in the epithelial cells of terminal bronchioles in the lungs, represents a target for autoantibodies in APECED (13). Indeed,in CIDP,the responsible autoantigens are unclear and the frequency of disease or the correlation with a specific phenotype are not reported. Recent studies with mouse models of autoimmune peripheral neuropathy strongly suggest a possible role of Myelin Protein Zero (PO) as the candidate autoantigen of CIDP.

This wide spectrum of the phenotype and the gradual appearance of symptoms over time, strongly suggest that, although APECED is the first well-documented example of an autoimmune disorder inherited as a monogenic disease, there are several functional, environmental or molecular factors which may contribute to the clinical expression of the disease. Associations with specific HLA aplotypes have been found for trait components like alopecia, Addison's disease and Type-1 Diabetes in Autoimmune Polyendocrine Syndrome 1 (APS 1). The associated haplotypes are those associated with the common, non-APS-1 related forms of these diseases. Only a weak association has been observed between the HLA type and autoantibody specificities in APS-1 patients, suggesting that in APS-1 the HLA alleles do not have a strong influence on autoantibody formation(15).

Along with the central tolerance network, which is primarily involved in pathogenesis of APECED, several other peripheral mechanisms are capable of contributing to the control and regulation of the immune system. These factors are involved in maintenance

of the homeostasis of peripheral tolerance of residual autoreactive clones which escape negative selection within the thymus and play a significant role in preventing or minimizing reactivity to self-antigens. The peripheral tolerance recognizes as possible mechanisms the induction of functional anergy, deletion of autoreactive clones by apoptosis and the suppressive action of regulatory T lymphocytes (Treg). Anergy is a mechanism that results in functional inactivation of self-reactive T cells. Clonal T-cell anergy can be induced upon engagement of the TCR in the absence of costimulatory signals. Apoptosis takes place through a series of regulated biochemical events that follow Fas/FasL interaction, thus resulting in cell death. Treg cells arise during the normal process of maturation within the thymus (16,17) and preferentially express high levels of CD25, the transcription factor forkhead box P3 (FoxP3) and a considerable number of additional activation surface markers, as transferrin receptor and HLA class II antigens. These cells exhibit a vast spectrum of autoimmunity-preventive activities (18). Treg cells are naturally anergic and, upon TCR activation, potently suppress the proliferation of CD4+CD25- T cells through an antigen-nonspecific mechanism. Moreover, the intimate molecular mechanism by which Treg cells mediate suppression still remains unclear (19). An additional mechanism involved in controlling reactivity to self engages in the periphery is Natural Killer (NK) cells activity. There is strong evidence that clearly shows the association between low levels of NK cells and NK-cell activity and the development of autoimmunity, attributable to failure in deletion of autoreactive clones by cytolysis. In this process, a pivotal role is played by protein such as Perforin (PRF1). Our group has documented that an alteration of the fine tuning of one of these processes is involved in the pathogenesis of autoimmune diseases (20-22). Therefore, alterations dependant on one of the peripheral tolerance mechanisms could contribute to the wide variability of APECED's clinical expression. To date, there are only few studies on the functionality of these immunological tolerance mechanisms in patients with APECED. Recently, a study has been published evaluating the number and functionality of Tregs in APECED patients (23). The authors reported an impairment of CD4+CD25+ Treg population in APECED patients, thus indicating that a Treg defect could be involved in the pathogenesis of APECED. However, the reduction in circulating Treg cells might also be secondary to the chronic fungal infection and autoimmune inflammation in these individuals. Therefore, performing Treg evaluation along with the study of other peripheral tolerance mechanisms at an early stage of the disease (e.g. in children) could help to clarify the role of these mechanisms in the

clinical phenotype of APECED patients. Another interesting aspect is the role that mutations of the AIRE gene have in the heterozygous subjects. AIRE gene mutations in a heterozygous state have also been identified in patients with hypoparathyroidism associated with Hashimoto's thyroiditis, in patients with systemic sclerosis associated with Hashimoto's Thyroiditis or in patients with sporadic Addison's disease (24-26). No data have been reported about AIRE status in patients affected with isolated chronic hypoparathyroidism. Furthermore, a recent study has been conducted in relatives of patients affected with APECED, heterozygous for AIRE mutations (10). These subjects were found to suffer from various autoimmune and non-autoimmune diseases but not major disease of APECED. In conclusion the effects of a slight impairment in the expression and/or function of AIRE protein, such as in the presence of heterozygous mutations of the gene, still need to be defined.

Aims of our project in this field are the followings:

A. **Genotypically and phenotypically characterize patients affected by Autoimmune Polyendrocrine Syndrome associated with a mutation of the AIRE gene through:**
   1. Definition of the modality of the disease's expression in pediatric age with particular attention to APECED atypical clinical manifestations including neurological involvement.
   2. Definition of the genotype-phenotype correlation.
   3. Study of the distribution of different genetic patterns based of the subjects' geographic area of origin.

B. **Study of environmental (infectivological triggers) and molecular factors that can presumably contribute to the phenotypical variability of the disease, with particular attention to the study of the peripheral tolerance mechanisms through the followings functional and molecular studies:**
   1. Evaluation of Natural Killer cells activity
   2. Evaluation of Fas-induced apoptosis in peripheral blood. mononuclear cells.
   3. Molecular analysis of Perforin gene mutations.
   4. Evaluation of T regulatory cells functionality and Foxp3 expression profile.

To these aims, we have genotipically and phenotypically characterized a population of pediatric patients affected with APECED originating from Campania. Within this population we identified a subject with an unusually severe phenotype of APECED

characterized by features never described before in association with the disease. Furthermore, we evalutad in this subject and in his sister, who presented only a mild phenotype despite the same molecular defect of AIRE, environmental factors and peripheral tolerance mechanimsms to establish wether these factors could be involved in the phenotypic intrafamiliar variability of APECED.

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3.2 Posterior Reversible Encephalopathy Syndrome in a child during an accelerated phase of a severe APECED phenotype due to an uncommon mutation of AIRE

Clinical Endocrinology (2008) **69**, 511–514

LETTERS TO THE EDITOR

Isolated R171Q amino acid change in *MEN1* gene: polymorphism or mutation?

The change of amino acid arginine to glutamine at position 171 (R171Q) in exon 3 of *MEN1* gene occurs in the general population with a frequency ranging from 1·4% to 5%, and has been occasionally reported in MEN1 carriers and in tissues from sporadic MEN1 endocrine tumours.[1–3] It is unclear whether an isolated R171Q amino acid change represents a polymorphism and/or it has a role in tumourigenesis.

Recently, Balogh *et al.*[4] reported the R171Q amino acid change in germline DNA of six out of 32 subjects with MEN1 or MEN1-related states. Specifically, the R171Q amino acid change was associated with three novel *MEN1* gene mutations (L301R, C354X and G28A) in two familial and one sporadic MEN1 cases, and was found to be the only *MEN1* gene alteration in other three subjects with sporadic MEN1-related state.

We report a case of a 46-year-old woman who came to our observation for recurrent primary hyperparathyroidism (PHPT). Three years before she underwent surgery for a parathyroid adenoma, a nonfunctioning pancreatic neuroendocrine tumour and a nonfunctioning adrenocortical adenoma. A MEN1 syndrome was diagnosed, but neither DNA analysis in the patient nor clinical investigations in the patient's relatives were available. Thus, studying family history in depth, we found that a few years earlier the patient's sister had undergone surgery for both a prolactin-secreting pituitary adenoma and PHPT due to parathyroid adenoma. The patient's father died for liver cirrhosis when he was 46 years old and during his life was affected by recurrent gastric ulcerations requiring gastrectomy. A patient's paternal aunt died for a cerebral mass, referred as a probable pituitary tumour causing amenorrhoea and blindness. We performed the *MEN1* germline gene analysis by direct DNA sequencing, as previously described,[5] in the proband, in the two sisters and in their brother. In all cases sequence analysis revealed an isolated G > A transition at codon 171 of exon 3, resulting in the replacement of arginine (CGG) with glutamine (CAG). Clinical investigation of the brother was negative for MEN1 features. The same gene alteration was also demonstrated in her brother's daughter, who had no clinical signs of disease.

The R171Q amino acid change was found in an unrelated MEN1 proband (affected by a parathyroid adenoma and an ACTH-secreting pituitary adenoma) from a different family, in her two sons (one with PHPT and a nonfunctioning pituitary adenoma) and in the father, not clinically affected. The relationship between the two MEN1 families studied was excluded by the study of genealogic trees.

Unfortunately, no tumour tissue from any of our patients was available to analyse the possible somatic loss of wild-type allele, in accordance with the Knudson two-hit hypothesis. As controls, a panel of 50 healthy Italian subjects, that is, 100 alleles, from the same geographical area was then screened, and the R171Q amino acid change in the *MEN1* gene was not detected. Written informed consent for genetic testing was obtained from all patients and control subjects.

More than 400 germline and somatic mutations have been identified in sporadic, familial MEN1 and MEN1-related states.[1–3] The *MEN1* gene screening is a complex and expensive procedure, and is justified only to confirm clinical diagnosis, to find other family carriers and to drive therapeutic decisions. Previous studies indicate the absence of mutational 'hot spots' in the *MEN1* gene and the lack of genotype–phenotype correlations in MEN1. On the basis of our results and of those of Balogh *et al.*[4] we suggest that the isolated R171Q amino acid change might be regarded as a point mutation with low penetrance MEN1 phenotype, rather than a harmless polymorphism. Therefore, MEN1 patients carrying this genetic alteration, as well as clinically unaffected carriers, should undergo a careful endocrine investigation and a close clinical and biochemical follow-up.

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Posterior reversible encephalopathy syndrome in a child during an accelerated phase of a severe APECED phenotype due to an uncommon mutation of AIRE

Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy syndrome (APECED) is an autosomal recessive disease, caused by mutations in the autoimmune regulator (AIRE) gene, affecting

many tissues, especially endocrine glands.[1] APECED is generally considered a slowly progressive disease.

We report on a child who developed an accelerated phase of a severe APECED phenotype culminating in a rare and life-threatening posterior reversible encephalopathy syndrome (PRES), when he was only 5 years old. Despite a wide variety of manifestations, PRES has never been described in APECED patients so far.

The proband, a boy from Southern Italy, first presented at the age of 5 years with severe asthenia, alopecia, and an urticaria-like erythema during a fever. His mother reported an episode of mild and transient hypertransaminasemia when he was 18 months old. Physical examination revealed the presence of vitiligo, nail dystrophy, oral candidiasis and hepatosplenomegaly. Laboratory investigations showed hypocalcaemia (1·3 mmol/l) and hyperphosphataemia (2·3 mmol/l) with undetectable levels of PTH, leading to the diagnosis of hypoparathyroidism. He was started on calcium and calcitriol therapy. Hypertransaminasemia (aspartate aminotransferase 476 U/l, alanine aminotransferase 495 U/l) was also present.

Over the next three months the patient showed marked and persistent fatigue. Biochemical tests revealed severe hypothyroidism (TSH 251 mIU/l, Free-T4 6·3 pmol/l) and he started L-T4 therapy. An increase in plasma renin levels (216 ng/l, normal range 1·8–33 ng/l) with normal cortisol response to ACTH stimuli was detected. Two months later, a blunted cortisol response to ACTH test confirmed biochemical adrenal insufficiency. He also presented gastrointestinal symptoms with abdominal distension accompanied by alternating diarrhoea and constipation. Coeliac disease and malabsorption were excluded. An extensive autoantibody study revealed a wide spectrum of autoantibodies (Abs) that corresponded to the clinical phenotype (Table 1). Autoimmune hepatitis (AIH) was scored as probable (score 12) on the basis of the recommendation of the international AIH group.[2] Thus, he was started on high-dose prednisone, in order to treat both hepatitis and glucocorticoid deficiency, and mineralcorticoid supplementation. Atrophic gastritis was diagnosed on the basis of persistently elevated levels of gastrinaemia (765 ng/l, normal range 0–80 ng/l), presence of parietal cells Abs (PCA) and histological findings of gastric body mucosa atrophy.

Table 1. Patient's clinical features and autoantibodies profile

Age (years)	Signs and symptoms	Autoantibodies against
1·5	Chronic hepatitis	AADC
5	Alopecia, urticaria-like erythema fever	
5·2	Vitiligo	CF-M
	Ectodermal dystrophy	
	Chronic hypoparathyroidism	
	Chronic mucocutaneous candidiasis	
5·5	Chronic thyroiditis with hypothyroidism	TM, Tg, TPO
	Addison's disease	21-OH, StCA, 17α-OH, p450scc
	Atrophic gastritis	PCA
	Abdominal bloating	TPH
5·8	PRES	

AADC, Aromatic L-amino acid decarboxylase; CF-M, melanocytes (complement-fixing); TM, thyroid microsomal; Tg, thyroglobulin; TPO, Thyroperoxidase; 21-OH, 21-hydroxylase; StCA, steroid producing cells; 17α-OH, 17α-hydroxylase; p450scc, P450 side chain cleavage; PCA, parietal cells; TPH, Tryptophan hydroxylase.

Because of a further increase in liver enzymes as well as side-effects of steroids, such as obesity and slight hypertension, he subsequently began to taper off steroids and started immunosuppressive therapy with azathioprine.

Six months after the onset of this accelerated phase, the patient suddenly developed severe symptomatology characterized by headache, mental confusion and transient blindness, culminating in lethargy and respiratory failure. Blood pressure was reported to be normal at hospital admission. Biochemical tests were unremarkable with normal calcium and magnesium levels. Magnetic Resonance Imaging (MRI) revealed hyperintense lesions on FLAIR and T2-weighted images involving both grey and white matter in parietal lobes (Fig. 1a). He did not receive any specific pharmacological intervention other than continuing to taper-off steroids. Thereafter, he experienced rapid and complete remission of neurological signs and an almost total resolution of the parietal lesions (Fig. 1b).

Direct sequencing of the patient's AIRE gene revealed the presence of a complex homozygous mutation in intron 1 consisting of a substitution of IVS1 + 1G by C accompanied by a single nucleotide deletion at IVS1 + 5G residue (IVS1 + 1G > C; IVS1 + 5delG). The parents were both heterozygous for the same mutation and had no features of autoimmunity.

In this study we report on a child who developed a severe APECED phenotype, with 10 symptoms breaking out over a 6-month period, culminating in life-threatening PRES. This is quite unusual when we consider that, although it usually begins in childhood, APECED has always been considered a slowly progressive disease and its full manifestation tends to occur over a period of years.[1]

PRES is a syndrome characterized by acute encephalopathy with specific radiological findings.[3] PRES is rare in childhood and has never been described in APECED patients so far. Affected patients typically experience headache, altered mental functioning, seizures, and visual loss associated with MRI images of white-matter oedema primarily involving the posterior parietal–temporal–occipital regions of the brain.[3] The pathophysiology of the syndrome is still unclear. It is associated with a variety of medical conditions, including hypertension, renal decompensation, fluid retention and treatment with immunosuppressants.[3] Oral steroid therapy has also been suspected as a causative factor in few cases.[4] PRES is usually reversible when promptly treated or if the triggering factor is removed. Left untreated, it can lead to irreversible damage.

Putative factors that may have triggered PRES in our patient were hypertension, steroids and azathioprine therapy. Although our patient's blood pressure was reported as normal upon being admitted to the hospital, it may have already peaked before admission. Furthermore, the appearance of PRES could be indirectly related to the use of steroids which may have provoked the presumed hypertension and fluid retention. Alternatively, the chronological association between the two may suggest the involvement of azathioprine as a trigger factor of PRES, even if a direct relationship between this drug and the development of the syndrome has never been clearly documented. However, the pathophysiology of PRES in

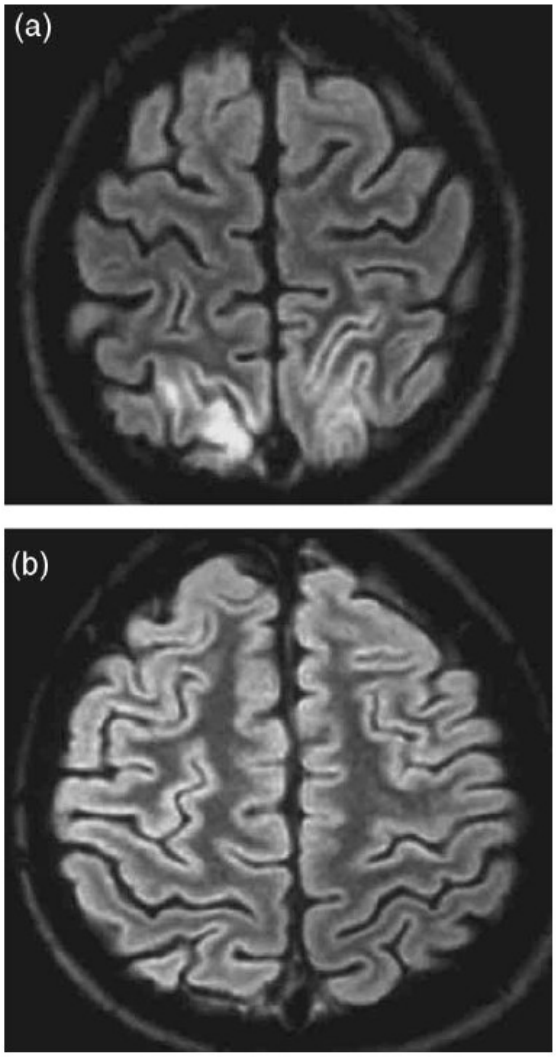

Fig. 1 Brain MRI scan. Increased signal in FLAIR involving both grey and white matter in parietal lobes (a) and almost complete resolution of the lesions after 1 month (b).

this case still remains unclear. It is noteworthy that our patient experienced complete remission of PRES without any specific pharmacological intervention, other than a slow tapering off of steroids.

Sequencing of the patient's AIRE gene revealed the presence of an uncommon homozygous mutation in intron 1. The sequence variant [IVS1 + 1G > C; IVS1 + 5delG] is made up of two mutations affecting the splicing in intron 1. The IVS1 + 1G is completely conserved in the major-class introns thus its change into C renders the splice site nonfunctional. The IVS1 + 5delG is also likely to have a negative effect because only G, A or T occur in the major-class introns at the IVS + 5 position, whereas the consequence of the deletion is a virtual G > C transversion.[5] To date, the IVS1 + 1G > C; IVS1 + 5delG mutation has never been described in homozygosity. It has been reported in a single individual from Poland in heterozygous state with R257X.[5] This patient only presented a mild phenotype during adolescence. Although, it is well known that there is no clear genotype–phenotype correlation for APECED patients, we cannot rule out that this mutation in homozygosity may be responsible for a severe phenotype of APECED or for the development of uncommon consequences.

In conclusion we report a child who, without any identified triggering factor, developed a very severe autoimmune polyendocrinopathy candidiasis ectodermal dystrophy syndrome phenotype characterized by an accelerated phase culminating in life-threatening posterior reversible encephalopathy syndrome. The management of such severe cases deserves further attention in order to identify novel therapeutic strategies for controlling such manifestations.

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3.3 High Intrafamiliar Variability in Autoimmune Polyendocrinopathy-Candidiasis Ectodermal Dystrophy: Study of the Peripheral Tolerance

PAPER IN PROGRESS

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Introduction

Context: Autoimmune Polyendocrinopathy Candidiasis Ectodermal Distrophy Syndrome (APECED) is an autosomal recessive disease, caused by mutations in the AutoImmune REgulator (AIRE) gene. Although APECED is a monogenic disease, clinical phenotype can reveal wide heterogeneity. This variability suggests that additional factors may influence the expression of the disease. Along with the central tolerance, primarily involved in pathogenesis of APECED, several other peripheral mechanisms contribute to regulation of immune system. So far, only a few study have investigated peripheral tolerance in APECED patients.

Objective: The aim of this study was to evaluate whether genetic, immunological and environmental factors may be involved in the modulation of the disease in two siblings with identical genotype and different phenotype.

Patients: Two siblings carrying the same complex homozygous mutation of AIRE (exon1: IVS1 + 1G>C; IVS1 + 5delG), showed a wide variability of phenotype: the older child (5 yrs) showed a severe phenotype complicated with uncommon manifestations, whereas the younger sister (4 yrs) had only a mild phenotype.

Methods: Perforin (PRF1) and HLA genes were amplified by PCR. APECED-related autoantibodies were performed by indirect immunofluorescence or complement fixation or ELISA or RIA. The following infectious markers were evaluated: Herpes, EBV, HZV, Parvovirus B19. Peripheral tolerance mechanisms were evaluated by: resistance to FAS-induced apoptosis on PBMC activated with PHA, the number of $T^{CD4+CD25+}$ regulatory cells (Treg) through cytometer analysis and NK activity through the Wallac method.

Results: No alteration was found in PRF1. HLA aplotype and exposure to infectious triggers were not apparently associated to the severity of the disease. Autoantibodies' profile paralleled in both siblings to the clinical manifestations. The evaluation of Treg was comparable in both children (1.51 and 1.05%, respectively), but lower as compared to controls (4.33%). The NK activity was comparable between the 2 siblings and the controls. FAS-induced apoptosis was normal in both children (75 and 80 % respectively, n.v. <82%).

Conclusions: Peripheral tolerance mechanisms and infectious triggers evaluated in the current study, as well as HLA aplotype, do not seem to play a role in modulating the phenotype of the two siblings. Further studies are needed to identify additional factors, other than AIRE gene mutation, involved in the phenotypic variability of APECED.

The genetic cause of many rare autoimmune diseases have been already identified and most of these disorders are a result of an intricate relationship between environmental and genetic factors, resultant in a deregulation of central and peripheral tolerance, that led to auto reactive pathogenetic T and B clones.

The intriguing evidence that the genetic background predisposes to the autoimmunity, but it doesn't define the specific target of the disease demonstrate that there are several factors that influence the phenotypical characteristics (1-4).

Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal-Distrophy (APECED; MIM 240300), or autoimmune polyglandular syndrome type I, is a rare autosomal recessive disease characterized by a set of three abnormal features: chronic mucocoutaneous candidiasis, hypoparathyroidism, and adrenal insufficiency. However, most patients also routinely exhibit a variable number of other autoimmune diseases. APECED is caused by mutations of a single gene, named autoimmune regulator (AIRE), which maps to 21q22.3 (5) and encodes a 55-kDa protein that acts as a transcription factor (6). Animal models of APECED have revealed that AIRE plays an important role in T cell tolerance induction in the thymus, mainly by promoting ectopic expression of a large repertoire of transcripts encoding proteins normally restricted to differentiated organs residing in the periphery. Thus, the absence of AIRE results in impaired clonal deletion of self-reactive thymocytes (7). Over 60 mutations have by now been localized in the AIRE genes of different APECED patients but the different mutations have not to date been convincingly associated with particular disease manifestations (7).

Although the disease is monogenic in transmission, APECED patients show a variable range of pathological manifestations, with each patient presenting a different constellation of affected organs and autoantibodies (autoAb) specificities. However, certain targets of the autoimmune attack are near constant, such as the adrenal and parathyroid glands, while other manifestations are less frequently observed (e.g., thyroiditis or T1D, which are present in 2–12% of cases) (8). Recent analysis have revealed that an effect of additional genetic loci, in particular the human leukocyte antigen (HLA) complex, is restricted only to few disease manifestations (9-10). Moreover, recent evidences indicate that also a defect in the regulatory T cells compartment (Treg) could be involved in the pathogenesis of APECED (11). So far, the reasons of the high phenotypical variability of APECED remain still unclear.

Our study was aimed to evaluate whether genetic, immunological and environmental factors may be involved in the modulation of the disease in two siblings, born from

consanguineous parents, with identical genotype and extremely different phenotype by studying the exposure to infectivological triggers, the HLA aplotype and several mechanisms of peripheral tolerance.

Subjects and Methods

Subjects

Case 1

The boy first presented at the age of 5 years with severe asthenia, alopecia, and an urticaria-like erythema during a fever. Physical examination revealed the presence of vitiligo, alopecia, nail dystrophy, oral candidiasis hypertransaminasemia and hepatosplenomegaly. Autoimmune hepatitis (AIH) was scored as probable (score 12) on the basis of the recommendation of the international AIH group.[2] Laboratory investigations showed hypocalcaemia (1.3 mmol/l) and hyperphosphatemia (2.3 mmol/l) with undetectable levels of PTH, leading to the diagnosis of hypoparathyroidism. He was started on calcium and calcitriol therapy. Over the next three months the patient developed autoimmune thyroiditis with severe hypothyroidism (TSH 251 mIU/L, Free-T4 6.3 pmol/l) and he started L-Thyroxine therapy. An increase in plasmatic renin levels (216 pg/ml, normal range 1.8-33 pg/ml) in the presence of adrenal autoantibodies, confirmed the diagnosis of adrenal insufficiency. He also presented gastrointestinal symptoms with abdominal distension accompanied by alternating diarrhea and constipation and presence of autonatibodies against TPH and AADC, commonly associated with gastrointestinal alterations in APECED patients. Atrophic gastritis was diagnosed on the basis of persistently elevated levels of gastrinemia (765 pg/ml, normal range 0-80 pg/ml), presence of parietal cells Abs (PCA) and histological findings of gastric body mucosa atrophy.

Six months after the onset of this accelerated phase, the patient suddenly developed a severe neurological symptomatology with neuroradiological findings suggestive of Posterior Reversible Encephalopathy Syndrome (PRES), a life-threatening event never described before in APECED patients.

Case 2

The girl, the younger sister of case 1, presented at the age of 4 years with hypoparathyroidism, diagnosed on the basis of hypocalcaemia, hyperphosphatemia and undetectable levels of PTH, and chronic mucocutaneous candidasis. No other signs or symptoms of APECED were revealed at physical examination or biochemical investaigations. During her follow-up, through the next two years, she did not develop any other features of the disease. Clinical details of the two siblings are shown in Table 1.

Molecular studies

Genomic DNA was extracted from peripheral blood. All 14 exons of the AIRE gene and exons 2-3 of the PRF1 gene were amplified with the use of primers located on the respective flanking introns (12) and then analysed by direct sequencing using the ABI PRISM 3130 sequencer (Applied Biosystems, Foster City, CA). The analysis included sequencing of the donor/acceptor sites of all of the introns.

Methods

The following autoantibodies were performed by classical indirect immunofluorescence technique or complement fixation or ELISA or RIA, as appropriate: Thyroglobulin Abs (TgAbs), Thyroid microsomal Abs (TMAbs), TSH-receptor Abs, Thyroperoxidase Abs (TPOAbs), Parietal cells Abs (PCA), Intrinsic factor Abs (IFA), Glutamic acid decarboxylase Abs (GADA), Adrenal cortex Abs (ACA), 17 α-hydroxilase Abs (17 α-OHAbs), Side-chain cleavage enzyme Abs (sccAbs), Aromatic-L-Aminoacid decarboxilase Abs (AADCAbs), Tryptophan hydroxylase Abs (TPHAbs).

The exposures to infectivological triggers, through the evaluation of specific immunoglobulins (Herpes, EBV, HZV, Parvovirus B19), the study of the HLA aplotypes and the analysis of Natural Killer (NK) cells activity were performed by standard procedures.

Cell death induced by Fas was evaluated as previously reported on T-cell lines obtained by activating peripheral blood mononuclear cells (PBMC) with phytohemagglutinin (PHA) at days 0 (1 μg/mL) and 15 (0.2 μg/mL) and cultured in RPMI 1640 1 10% FCS1recombinant IL-2 (5 U/mL) (Biogen, Geneva, Switzerland). Fas function was assessed 6 days after the second stimulation (day-21 T cells).

The number of Treg cells was evaluated by flow cytometry. Peripheral blood mononuclear cells (PBMC) (1*106) were treated with antibodies against CD4-FITC (RPA-T4 eBiosciences) and CD25-APC (BC96 eBiosciences) and then analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences).

For the proliferation assay, PBMC ($2*10^5$ cell/200 µl well) were cultured triplicate in 96-well Ubottom microtiter plates (Falcon; BD Biosciences) with or without phytohemagglutinin (PHA) at reported concentrations for 4 days. The proliferative response was evaluated by thymidine uptake from cultured cells pulsed with 0.5 µCi of [^{3}H]-thymidine (Amersham Biosciences) 8 h before harvesting.

Results

Direct sequencing of the patients' *AIRE* gene revealed in both the siblings the presence of the same complex homozygous mutation in intron 1 consisting of a substitution of IVS1 + 1G by C accompanied by a single nucleotide deletion at IVS1 + 5G residue (IVS1 + 1G>C; IVS1 + 5delG). The parents were both heterozygous for the same mutation and had no features of autoimmunity. This mutation represents an uncommon mutation of AIRE and, so far, has been described in heterozygous state with the R257X in a single individual from Poland.

As previuosly described, circulating autoantibodies paralleleld in each patient the clinical phenotype (Table 2), and strongly supporting the autoimmune mechanism at the basis of the pathogenesis of APECED. Moreover, the evaluation of specific response to infectivological triggers, showed no substantial differences that could influence the severity of the disease in that the younger sister, which presented a milder phenotype, was more exposed to viral infections.

To define the possible contribution of distinct peripheral tolerance mechanisms in the high variability observe in the two patients, we also analyzed the resistance to Fas-induced apoptosis and the number of CD4+CD25+ cells indicative of the presence of Treg population. In particular, cell death assay after Fas stimulation revealed that the resistance to Fas-induced apoptosis was comparable between the siblings and normal as in the controls. Flow cytometric assays revealed a comparable number of CD4+CD25+ T cells in the two siblings, but in both cases reduced as compared to controls.

Being NK cells involved in the autoimmunity attributable to failure in deletion by cytolitic protein such as Perforin (PRF1), we analyzed NK cells activity that resulted comparable to controls in both the cases. Moreover, no mutations in the coding region

of *PRF1* gene were identified in both the siblings. It was notable that the molecular study of the *PRF1* gene disclosed a heterozygous nucleotide substitution in *PRF1* exon 3 resulted in the Ala273Ala silent mutation. This substitution was only found in the Case 1.

Discussion

Here we report the case of two siblings affected with APECED that showed an high intra familiar variability of phenotype despite the same genotype of the AIRE gene. Although APECED is generally considered a monogenic disorder, it is characterized by a wide variability of expression with each patient presenting a different constellation of affected organs and autoAb specificities.

As other autoimmunity disease, APECED is the result of alterations at different levels, as demonstrated by the remarkable north/south gradient in the susceptibility to autoimmune diseases, that suggests that environmental factors could be involved (13).

Studies of *Aire* knockout mice have provided direct evidence that Aire has a vital role in preventing autoimmunity. In particular, APECED animal models revealed that Aire plays an important role in T-cell tolerance induction in the thymus, mainly by promoting ectopic expression of a large repertoire of transcripts encoding proteins normally restricted to differentiated organs residing in the periphery. Thus, the absence of AIRE results in impaired clonal deletion of self-reactive thymocytes, which escape into the periphery and attack a variety of organs (7).

The extreme variability of APECED presentation, even between siblings with the same genotype, lead to speculate that other mechanisms, beyond aire itself, could be involved in the clinical expression of the disease.

We observed two siblings patients with the same genetic AIRE mutations, but with a spectrum of target organs completely different. Although it is well known that APECED is characterized by extreme variability of phenotype, the reasons of this phenomenon remain still unclear.

In particular, being the infectious agents represent a potent stimulus for the immune system and may contribute to select auto reactive T cells in susceptible subjects, through molecular mimicry (14-16), bystander activation (17-18) and epitope spreading (19), we examined the associated infections in both patients, but they show an identical infective story. Moreover, we analyzed NK activity and eventual genetic alterations in PRF1 gene. The results of these analysis showed a comparable NK

activity any alterations in *PRF1* gene for both patients. It is likely that a nonscreened mutation in the intronic or in the promoter region of *PRF1* gene could influence the different phenotype observed in the siblings.

Furthermore, on the other side of the central tolerance there are other mechanisms that maintain tolerance to self, such as the induction of functional anergy, deletion by apoptosis, and the suppressive actions of Treg. On the bases of these mechanisms we analyzed in particular the resistance to apoptosis by cell death assay with Fas, the number and the functionality of Treg, attributable to a normal expression of the transcription factor Foxp3. During this analysis we didn't note any variations between the two sibling patients.

In summary, we found a high intrafamiliar variability in two siblings affected with the same mutation of *AIRE* gene. The results imply that the analyzed mechanisms did not influence the phenotypic spectrum of APECED.

Disclosure Statement

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Table 1 Clinical expression of the disease in the two siblings

Symptoms and signs	**Case1**	**Case 2**
Hepatitis	**x**	**-**
Hypoparathyroidism	**x**	**x**
Candidiasis	**x**	**x**
Alopecia	**x**	**-**
Vitiligo	**x**	**-**
Ectodermal Dystrophy	**x**	**-**
Addison	**x**	**-**
Urticaria-like erythema-fever	**x**	**-**
Hypothyroidism	**x**	**-**
Atrophic Gastritis	**x**	**-**
Abdominal Bloating	**x**	**-**
PRES	**x**	**-**

Table 2 Autoantibodies profile in the two siblings

Autioantibodies against	**Case 1**	**Case 2**
21-OH	**X**	**-**
PC	**X**	**-**
Tg	**X**	**-**
TPO	**X**	**-**
StCA	**X**	**-**
17αOH	**X**	**-**
TPH	**X**	**-**
scc	**X**	**-**
CF-m	**X**	**-**
AADC	**X**	**-**
ACA	**X**	**-**

3.4: MOLECULAR DEFECTS IN THE AIRE GENE IN APECED PATIENTS FROM CAMPANIA: HIGH PREVALENCE OF EXON 1 MUTATIONS.

PAPER SUBMITTED

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Short title: AIRE mutations in APECED patients from Campania

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ABSTRACT

Background: Autoimmune Polyendocrinopathy Candidiasis Ectodermal Distrophy Syndrome (APECED) is an autosomal recessive disease, caused by mutations in the AutoImmune REgulator (AIRE) gene. The prevalence of APECED worldwide is very low. Some different mutations have been identified to be peculiar to particular populations. In Italy the disease is also very rare but three hot spots areas have been identify. In Sardinia the typical AIRE mutation is characterized by a nonsense mutation on exon 3, defined as R139X. In Apulia a typical AIRE mutation is localized on exon 2 and is defined as W78R. In northern Italian populations R257X is very frequent and often associated with 1094-1106del113 and in Sicily a typical mutation defined R203X has been recently suggested. As regard to the Campania region only one patient has been studied and the mutations identified are on the exon 11.

Aim: In this study we carried out mutation analysis of the AIRE gene in 6 patients affected with APECED originating from the region of Campania, an area of Southern Italy.

Patients and methods: Six children, originting from 5 different families of the region of Campania, were diagnosed as having APECED on the basis of the presence of at least two of the three major signs of the disease. Genomic DNA was estracted from peripheral leukocytes and the 14 exons of AIRE were amplified by PCR. A complete assessment of APECED-related autoantibodies' was performed by classical indirect immunofluorescence technique or complement fixation or ELISA or RIA, as appropriate.

Results: In all patients mutational analysis confirmed the diagnosis of APECED. All patients carried at least one mutation on exon 1:

- two siblings carried a complex homozygous mutation [IVS1 + 1G>C; IVS1 + 5delG] on intron 1;
- two patients were compound heterozygous for [T16M]+[W78R] (exons 1+2);
- one patient was compound heterozygous for [A21V]+[C322fs] (exons 1+8);
- one patient was homozygous for [T16M]+[T16M] on exon1

The phenotypic expression of the disease showed wide variability, even between siblings with the same genotype. Circulating auto antibodies paralleled to the clinical symptoms in each patient.

Conclusion: Mutations on exon 1, in homozigosity or compound heterozygosisty, seem to be highly frequent in patients originating from Campania region. Although there is not a single typical mutation, the exon 1 could be suspected to represent a "hot spot" region for APECED patients originating from Campania. As already reported, genotype-phenotype analysis failed to reveal a clear genotype-phenotype correlation.

INTRODUCTION

Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal-Distrophy (APECED) is a rare autosomal recessive disease (OMIM 240300) which affects many tissues especially endocrine glands (1). The diagnosis is primarily based on the presence of two out of the three most common clinical features: hypoparathyroidism, Addison's disease, and chronic mucocutaneous candidiasis (1). Chronic mucocoutaneous candidiasis is often the first clinical manifestation to appear before the age of 5 year, followed by hypoparathyroidism and later by Addison's disease. APECED is caused by mutations in the AutoImmune REgulator gene (AIRE), which maps to 21q22.3 (2, 3) and encodes a 55-kDa protein that acts as a transcription regulator (5). Over 60 mutations have by now been localized in the AIRE genes of different APECED patients (5).

Even though it occurs through the world, its incidence is higher in some genetically isolated populations. The estimated prevalence of APECED is 1:9.000 in Iranian Jewes (6) 1:25.000 in Finns (7,8) and 1:14.400 in Sardinians (9). Some different mutations have been found to be peculiar of specific areas. R257 X is the most common mutation among Finnish patients (10), 1094-1106del113 (or 967-979del13 bp) is the most common mutation in British (11), Irish (13), North America (13, 14) and Norwegian patients (15) and the Y85C mutation is more frequent among Iranian Jews (16).

The different mutations have not to date been convincingly associated with particular disease manifestations (5).

In Italy three hot spots areas can be identified: Sardinia, Apulia and a small town (Bassano del Grappa) in the Venetian region. Furthermore, a peculiar mutation of AIRE has been identified in Sardinia and Apulia: the R139X mutation on exon 3 in Sardinia (17) and the W78R mutation on exon 2 in Apulia (18). Recently studying three patients with APS-1 from Sicily (19, 20) it was suggested the existence of a typical mutation of this region characterized by R203 X on exon 5.

So far only one patient with APECED was descry.bed from Campania having the mutation on exon 11 (21).

Aim of our study was to characterize the clinical presentation, the autoantibodies' production and the molecular defects of AIRE in 6 pediatric patients affected with APECED originating from Campania.

Patients

Six patients affected with APECED (5 F, 1 M) originating from Campania, a region of Southern Italy, were investigated. The patients were originating from 5 unrelated families. One of the 5 family have two children affected and all the other families have one affected child. Consanguinity between families was documented in two families: in both families parents were third cousins.

Diagnoses of the different clinical manifestations were made accordingly to established criteria (1). The onset of the disease and the clinical manifestations of for each patient are shown in Table 1.

Mutation detection

Genomic DNA was extracted from peripheral blood. All 14 exons of the AIRE gene were amplified with the use of primers located on the respective flanking introns (16) and were analysed by direct sequencing using the ABI PRISM 3130 sequencer (Applied Biosystems, Foster City, CA). The analysis included sequencing of the donor/acceptor sites of all of the introns.

Autoantibodies' production

The following autoantibodies were performed by classical indirect immunofluorescence technique or complement fixation or ELISA or RIA, as appropriate: Thyroglobulin Abs (TgAbs), Thyroid microsomal Abs (TMAbs), TSH-receptor Abs, Thyroperoxidase Abs (TPOAbs), Parietal cells Abs (PCA), Intrinsic factor Abs (IFA), Glutamic acid decarboxylase Abs (GADA), Adrenal cortex Abs (ACA), 17 α-hydroxilase Abs (17 α-OHAbs), Side-chain cleavage enzyme Abs (scabs), Aromatic-L-Aminoacid decarboxilase Abs (AADCAbs), Tryptophan hydroxylase Abs (TPHAbs).

RESULTS

Patients

As shown in table 1, in all patients the onset of symptoms was very early. All patients but one had at least two of the three classic symptoms at diagnosis. The one patient with only one classic symptom presented at the age of 0.7 years with vasculitis, then followed by candidiasis at the age of 2 years. Only 3 patients presented the classic tryad during follow-

up. The severity of the disease was widely different and many patients experienced atypical manifestations while in others the phenotype was mild. In particular, one patient developed a lifethreatening posterior reversible encephalopathy syndrome (PRES) that has never been previously described in the context of APECED syndromes (22), and another patient presented with only vasculitis at an early age, as above decribed. Interestingly, in 3 of the 4 patients with Addison, the disease appeared in early childhood, whereas it is usually reported in the second decade.

AIRE mutation analysis

Four different mutations were detected (Table 2). None of the mutation described was a novel mutation and the complex variant [IVS1 + 1G>C; IVS1 + 5delG] is uncommon. Interestingly, all patients carried at least one mutation on exon 1 and out of 12 alleles, nine (12%) showed a mutation of AIRE localized on the exon 1 (Table 2). Two siblings carried a complex homozygous mutation in intron 1, consisting of a substitution of IVS1 + 1G by C accompanied in *cis* by a single nucleotide deletion at IVS1 + 5G residue [IVS1 + 1G>C; IVS1 + 5delG]. Two patients were compound heterozygous for [T16M]+[W78R] on exons 1 and 2; one patient was compound heterozygous for [A21V]+[C322fs] on exons 1 and 8 and one patient was homozygous for [T16M] +[T16M] on exon 1.

The sequence variant [IVS1 + 1G>C; IVS1 + 5delG] consists of two mutations, each likely to affect the splicing of intron 1. The IVS1 + 1G is 100% conserved in the major-class introns so that its change into C must render the splice site nonfunctional. The IVS1 + 5delG is also likely to have a negative effect because in the major-class introns at the IVS+5 position only G,A or T occur.

Mutations T16M, A21V and W78R are localized in the homogeneously staining region (HSR) domain of AIRE, which is the area in which most other missense mutations have been located. Missense mutations and also small deletions affecting the HSR domain of the AIRE protein lead to the production of a functionally defective protein due to the loss of its homodimerization propertie.

Autoantibodies' profile

As previuosly described, circulating autoantibodies paralleleld in each patient the clinical phenotype (Table 3), strongly supporting the autoimmune mechanism at the basis of the pathogenesis of APECED.

DISCUSSION

In this study we have delineated the molecular pathology and the clinical spectrum of 6 probands affected with APECED originating from the region Campania of the Southern Italy. Our results suggest that in Campania an hot spots area of mutations of AIRE can be identified and it is localized on exon I of the gene.

World-wide prevalence of APS type 1 is very low; however, among the Iranian Jewish (6), in Finland (7,8) and in Sardinia (9) the estimated prevalence is 1/9,000, 1/25,000, and 1/14,000 respectively. A higher prevalence of APS type 1 among some populations could be related to a founder effect gene (1).

Only a few studies have been conducted to delineate the molecular pathology and phenotype of APECED patients originating from Italy. However, recent studies have documented that three hot spots area can be identified in Italy. The first is Sardinia, where the APECED prevalence is 1:14,400 (9); the second is Apulia with a frequency of 1: 35,000 (18) and the third is the Venetian region with a frequency of 1:4,400 (23). Moreover, AIRE typical mutations have been identified both in Sardinia (R139X) (9) and in Apulia (W78R) (18). A recent study on Italian APECED patients identified a possible typical mutation also in Sicilian patients, named R203X and localized on exon 5, whereas failed to reveal a typical mutation in patients originating from North of Italy or Venice. However, even if a typical mutation is not present in the Nothern Italy and Venetian populations, two mutations, already described in European populations characterize the AIRE genetic pattern of this Italian area (20).

Our study suggest that the frequency of AIRE mutations in Campania seems to be relatively high. Interestingly, we found that mutations localized on exon 1, being present either in compound heterozygosity or homozygosity, are relatively common in APECED patients originating from Campania. In fact, out of 12 alleles from 6 different probands, 9 (75%) showed mutations on exon 1, suggesting that, although a single typical mutation cannot be identified, this region of the gene could represent an hot spots area of mutation in this area of Southern Italy.

None of the mutations detected in our patients was novel, however they were all uncommon . In particular 2 siblings carried a complex homozygous mutation that consists of two mutations affecting the splicing in intron 1. This mutation has never been reported in APECED patients from Italy. So far, the IVS1 + 1G>C; IVS1 + 5delG mutation has been described in heterozygous state with the R257X in a single individual from Poland (24).

The analysis of genotype-phenotype in our subjects correlation failed to reveal a clear relationship, as previously reported in other series of patients. The presenting symptoms, the age of onset and the phenotype at the last visit were extremely different between our patients even when the genotype was the same. In particular the 2 siblings carrying the same complex homozygous mutation showed a wide heterogeneity of clinical expression: one patient

developed a severe phenotype culminating in a life-threatening event, whereas his sister presented with only a mild phenotype. Patient 2 and 5, presenting the same mutation also widely differed in their phenotype: the first patient, in fact, had only a mild phenotype with a few symptoms, whereas the second patient developed a severe phenotype since the first decade of life. Moreover, the early development of Addison disease was also not apparently related to the genotype in that the three patients affected with Addison within the first decade of life carried different mutations of exon I.

In conclusion our data demonstrate that mutations on exon I of AIRE gene are common in APECED patients from Campania, suggesting that not only in Sardinia, Apulia and Sicily but also in Campania an hot spots area of mutations can be identified. Further studies on large number of patients are needed to better evaluate the frequency of exon I mutations in Campania and its effect on APECED phenotype.

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Table 1. Clinical manifestations and age of onset of 6 APECED patients from Campania

Patient	**1**	**2**	**3**	**4**	**5**	**6**
Age at onset (yr)	0.7	4	1.5	2	2	6
Age at the last visit	6	7	8	8	28	20
Mucocoutaneous Chronic Candidiasis (CMC)	+	+	+	+	+	-
Hypoparathyroidism (HP)	-	+	+	+	+	+
Addison's disease (AD)	-	-	+	+	+	+
Hypogonadism (HH)	-	-	-	-	+	-
Diabetes Mellitus (IDDM)	-	-	-	-	-	-
Autoimmune Thyroiditis (AT)	+	-	+	-	+	-
Atrophic gastritis (AG)	nt	nt	+	nt	nt	nt
Pernicious anemia (PA)	-	-	-	-	-	-
Chronic hepatitis (CH)	-	-	+	-	-	-
Malabsorption	-	-	+	-	+	-
Keratitis	-	-	-	-	-	-
Alopecia (A)	+	-	+	-	-	+
Vitiligo (V)	-	-	+	-	-	-
Ectodermal Distrophy (ED)	-	-	+	-	+	-
Enamel hypoplasia	-	-	-	-	+	+
Vasculitis	+	-	-	-	-	-
Parotitis	-	-	+	-	-	-
Pancreatitis	-	-	-	-	+	-
Urticaria-like erythema fever	-	-	+	-	-	-
Abdominal bloating	+	-	+	-	+	-
Posterior Reversible Encephalopaty syndrome (PRES)	-	-	+	-	-	-

Table 2. Mutation of AIRE gene in six APECED from Campania

Patient	AIRE Genotype	Exons
1	47C>T / 232 T>A	I/II
2	IVS1 + 1G>C, +5delG / IVS1 + 1G>C, +5delG	I/I
3	IVS1 + 1G>C, +5delG / IVS1 + 1G>C, +5delG	I/I
4	62C>T / 967-979 del	I/VIII
5	47 C>T / 232 T>C	I/II
6	T16M/T16M ?	I/I

Table 3. Main Autoantibodies screened in the 6 APECED patients from Campania

Patient	1	2	3	4	5	6
Thyroglobulin Abs (TgAbs)	+	-	+	-	+	-
Thyroidmicrosomal Abs (TMAbs)	+	-	+	-	+	-
TSH-receptor Abs	nt	nt	-	nt	nt	nt
Thyroperoxydase Abs (TPOAbs)	-	-	+	-	+	nt
Parietal cells Abs(PCA)	+	-	+	-	-	-
Intrinsic factor Abs(IFA)	nt	-	-	-	-	-
Islet-cell Abs (ICA)	nt	-	-	-	+	-
Glutamicacid decarboxylase Abs (GADA)	-	-	-	-	nt	-
Adrenal cortex Abs (ACA)	+	-	+	-	+	+
21-hydroxylase Abs (21-OHAbs)	nt	-	+	+	+	+
Steroid-producing cells Abs (StCA)	nt	-	+	-	+	+
17 α-hydroxylase Abs (17 α-OHAbs)	nt	-	+	-	+	-
Side-chain cleavage enzyme Abs (sccAbs)	nt	-	-	-	+	+
Aromatic-L-aminoacid decarboxilase Abs (AADCAbs)	nt	-	+	-	+	-
Tryptophan hydroxylase Abs (TPHAbs)	nt	-	+	-	+	-
MPCA (melanin-producing cells Abs)	nt	-	+	-	-	-

nt=not tested

CONCLUSIVE REMARKS

In the context of autoimmune diseases as model of complex diseases involving both endocrine and autoimmune systems, we paid particular attention to the study of the Polyglandular Autoimmune Syndrome type I or APECED.

We characterized the molecular defects and the phenotype of a group of 6 pediatric patients originating from the region of Campania. Our results document that mutations on exon I of AIRE gene are common in APECED patients originating from Campania and suggest that in this region an hot spot of mutations of AIRE can be identified such as previously described in other regions of Italy. As reported for other populations, also in our patients we did not find a correlation between phenotype and genotype and the clinical expression of the disease wide varies even between patients with the same genotype. We described a couple of siblings with same genotype and an extremely different phenotype. The older brother was particularly interesting also because he developed an unusually severe phenotype complicated by a life-threatening encephalopathy never described before in APECED patients. Furthermore, we compared in these two siblings exposures to infectivological (viral infections) and peripheral tolerance mechanisms to evaluate wheteher the familial variability of APECED could be related to a different infectivological exposure or alterations in immunological peripheral tolerance. We did not find significative differences between the two siblings and thus our preliminary results suggest that these mechanisms are not involved in the modulaton of the severity of the disease.

CHAPTER 4
SHARED SIGNALING BETWEEN IMMUNE AND ENDOCRINE SYSTEM

4.1 Introduction and aims

Endocrine and immune systems participate to an integrated network of soluble mediators that communicate and coordinate responsive cells to achieve effective functions in an appropriate fashion. Cytokines and growth factors transmit signals through cell-surface receptors to the nucleus, activating intracytoplasmatic signaling molecules, ultimately resulting in the activation of specific transcription factors. In the recent years, the description of complex phenotypes, in which immunodeficiency and growth failure were associated at a different extent greatly contributed to define that several signaling molecules play a role in both Growth Hormone (GH)-related and cytokines' signaling pathways. In fact, mutations of gamma chain (γc), Signal Transducers and Activators of Transcription 5 b (STAT5b), Nuclear Factor-kB (NF-kB) gene have been observed in patients with short stature due to GH insensivity (GHI) and immunodeficiences (1-7).

Recently, mutations of the STA5b gene have been demonstrated in patients affected with complex phenotypes involving both endocrine and immune systems. STAT5b is a shared component between signalling pathways implicated in both immunological and endocrine functions and can be activated by many cytokines, IL-2, IL-7, IL-21 and IFN-γ.

To date, a total of six cases of growth failure associated with genetic abnormalities of STAT5b have been identified (8-10). The clinical pheotpye of these patients were characterized by growth failure and immunodeficiency. Recurrent pulmonary infections, chronic diarrhoea, severe eczema, herpes keratitis, severe varicella, juvenile arthritis, lymphoid interstitial pneumonia with fibrosis, were reported in these patients. Further studies in these patients revealed several immune deficiencies, such as decreased number and function of CD4+CD25 high regulatory T cells, low numbers of NK and γδ T cells, and IL-2 signaling abnormalities (11). However, the relationship between endocrine and immune dysfunctions in patients with STAT5b alterations are not yet completely defined.

Moreover, recently Adriani et al demonstrated that the common cytokine receptor γc is required for a proper GH mediated STATA5b activation in B cell lines (BCLs) (12), suggesting a novel dependence of GH signaling on the common cytokines receptor γc in certain cell types.

Specific aim of this review was to focus on the multiple roles in haematopoietic and non-hematopoietic receptors of the gamma signaling element with a special attention paid to the participation of gamma to growth hormone receptor signaling, confirming the presence of an interplay between endocrine and immune system.

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4.2 SHARED SIGNALING PATHWAYS BETWEEN ENDOCRINE AND IMMUNE SYSTEM RECEPTORS: THE MODEL OF GAMMA CHAIN

Current Signal Transduction Therapy, 2008, *3*, 000-000

Shared Signaling Pathways Between Endocrine and Immune System Receptors: the Model of Gamma Chain

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Abstract: The rapid expansion in the past two decades in the understanding of the molecular basis of a large variety of novel congenital immunodeficiencies has provided valuable information on the signal transduction general mechanisms, that goes far beyond the comprehension of the individual disease. In most cases, the altered molecules are exclusively expressed in haematopoietic cells, while in other cases they are not restricted to a certain cell type. This leads to more complex clinical phenotypes, which contribute to unravel previously unappreciated non-haematopoietic functions of signaling proteins and the mechanism of coordination and integration of several pathways. Moreover, this knowledge will help define potential new therapeutic strategies through novel molecular targets, drive stem cell development into the desired differentiation path and ameliorate our comprehension of tissue engineering. This review focuses on the multiple roles in haematopoietic and non-haematopoietic receptors of the gamma signaling element with a special attention paid to the participation of gamma to growth hormone receptor signaling, confirming the presence of an interplay between endocrine and immune system.

Key Words: Congenital immunodeficiencies, growth hormone receptor, signal transduction, gamma chain, STATs.

1. INTRODUCTION

Endocrine and immune systems participate to an integrated network of soluble mediators that communicate and coordinate responsive cells to achieve effective functions in an appropriate fashion. Cytokines and growth factors transmit signals through cell-surface receptors to the nucleus, activating intracytoplasmatic signaling molecules, ultimately resulting in the activation of specific transcription factors.

In the recent years, the description of complex phenotypes, in which immunodeficiency and growth failure were associated at a different extent greatly contributed to define that several signaling molecules play a role in both Growth Hormone (GH)-related and cytokines' signaling pathways. In fact, mutations of gamma chain (γc), Signal Transducers and Activators of Transcription 5 b (STAT5b), Nuclear Factor-κB (NF-κB) gene have been observed in patients with short stature due to GH insensitivity (GHI) and immunodeficiencies [1-7].

Recently, Adriani *et al.* demonstrated that the common cytokine receptor γc is required for a proper GH mediated STAT5b activation in B cell lines (BCLs) [8]. This study suggests a novel dependence of GH signaling on the common cytokines receptor γc in certain cell types, consistent with the presence of γc in non-hematopoietic tissues and underlines that immune and endocrine systems share signaling molecules.

This review, taking advantage of these complex human disorders, will focus on the relationship between different receptors that share common transducing elements and on potential diagnostic and therapeutical implications of such interactions.

2. THE GAMMA CYTOKINE TRANSDUCING ELEMENT, A SHARED COMPONENT OF SEVERAL CYTOKINE RECEPTORS

A wide number of cytokines have been molecularly recognized. They are soluble elements that control the immune and the haematopoietic system [9]. Their pleiotropic and redundant functions are due to the various receptors expressed on multiple target cells. Cytokines' specific roles are, in fact, closely dependent on their recognized targets. A contribution to a better understanding of such cytokine functions came out from the molecular characterization of a number of cytokine receptors. These receptors are classified into five families, created on the basis of extra- and intra-cellular domains structure affinity: the cytokine receptor superfamily, Interferon (IFN) receptor family, Tumor Necrosis Factor (TNF) receptor family, Tumor Growth Factor- (TGF)-β receptor family, and Interleukin (IL)- 8 receptor family. The cytokine receptor superfamily is the largest one, containing not less than 18 different receptor molecules. The common cytokine receptor γc is a shared component of several of these receptors, such as those for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 [9]. Essentially, the γc and gp130 molecules contribute to increase the ligand-binding affinity and to establish an efficient intracellular signal transduction (Fig. **1**).

Most of the information so far available on the role of γc came out from studies on X-Linked Severe Combined Immunodeficiency (SCID-X1) in humans and in mice carrying mutations in the γ gene [10,9,11].

The signals, that are dependent on γc, have been shown to mediate various immunological functions. The characterization of cytokine-activated genes, including genes regulated by γc-dependent cytokines, has been for a long time an area of considerable interest, although only few systematic studies have been conducted [12].

Even if the role of different cytokine-dependent signaling pathways in the regulation of gene responses is still unresolved, there is substantial evidence that γc controls the immune response at different, but often overlapping, checkpoints. IL-2, IL-7, and IL-15 were reported to regulate a highly overlapping set of more than one hundred overlapping genes, exerting different effects correlated to T-cell survival, activation and clonal expansion, and to the development and preservation of cell memory [13]. Moreover, IL-7 regulates lymphocyte development and homeostasis and it has effects on both T- and B-cell biology [14-16]. Although IL-7 receptor (IL-7R) is highly expressed on resting T cells, it is rapidly down-regulated by T-cell receptor (TCR) stimulation as well as by IL-2, thus supporting the idea that other cytokines are more relevant for effector functions [17]. In particular, IL-2 and IL-15 exert an important effect on promoting T-cell growth *in vitro*. In the effector phase of immune response, they can stimulate the proliferation of Natural Killer (NK) cells as well as induce the NK cell cytolytic activity. However, an important difference in the actions of these two cytokines has emerged *in vivo*, in particular, with regard to NK-cell development and $CD8^+$ T-cell homeostasis [18]. In fact, mice deficient in IL-2Ra or IL-2Rb have almost normal number of T, B and NK cells, thus indicating that IL-2 does not play a necessary role in cell development. Indeed, mice deficient in either IL-15 or IL-15Ra lack NK cells, confirming the role of IL-15 in NK-cell development [19,20]. Similarly, IL-15 is essential for the homeostatic prolifera-

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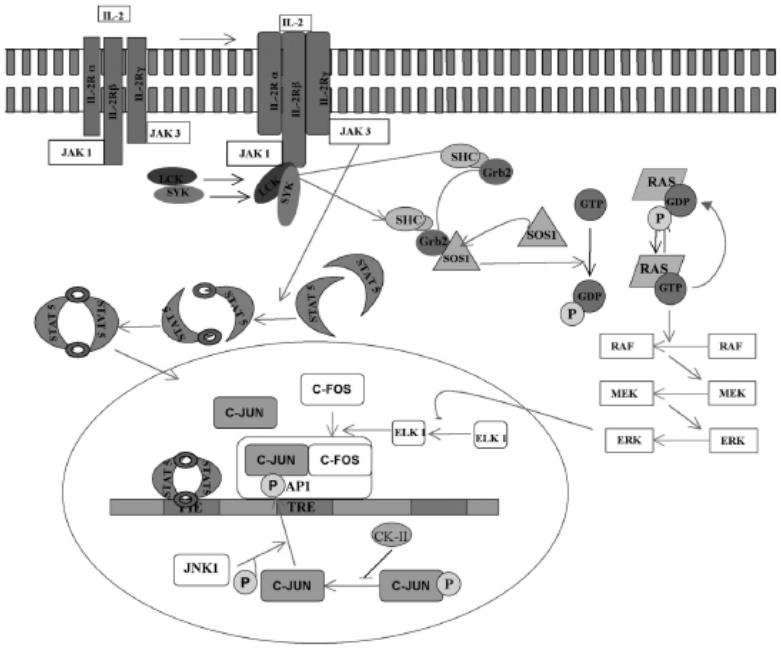


Fig. (1). Signaling pathway of IL-2 receptor.

tion of memory $CD8^+$ T cells and the maintenance of the steady-state level of $CD8^+$ T-cell memory.

IL-9 is a mast-cell growth factor. In IL-9-deficient mice, the lymphoid compartment develops normally. However, these mice exhibit excessive mucus production and mast cell proliferation [21]. But, such abnormalities have not been reported in humans with SCID-X1, suggesting that the γc is not responsible *per se* of this phenomenon.

Gene knockout (KO) models of γc-containing receptors for IL-4 and IL-21 have revealed a role in the regulation of immunoglobulin (Ig) production. Previous studies indicated a role for IL-4 in B-cell Ig class-switch to IgG1 and IgE [22].

It is now clear that cytokines acting through γc-containing receptors regulate several aspects of immune activation and play an important role in supporting either survival and proliferation or effector functions of already activated immune cells. To date, the regulation of cell survival and apoptosis can be considered a delicate teamwork and a balanced act of all γc-dependent cytokines seems to play a crucial role, as revealed by the profound impact that their abnormal function can have on the homeostasis of the immune system.

The most important function shared between all γc-containing receptors seems to be the mitogenic effect. It is presumable that, given the widespread use of γc in different tissues, this pathway exerts its mitogenic and anti-apoptotic functions in a variety of distinct contexts (Table **1**) [23].

3. PARTICIPATION OF γC TO GH-R SIGNALING

Recent evidence indicates that γc participates to growth hormone receptor (GH-R) signaling, as well [8]. A previous observation was reported on a patient affected with SCID-X1 due to mutation of γc, who also had peripheral GH hypo-responsiveness associated with abnormalities in the protein phosphorylation events that normally occur following GH-R stimulation [7]. This observation, along with the abundance of γc in non-haematopoietic tissues, led

Table 1. Main Immunological Features of γc-Dependent Cytokines/Receptors

	Knockout Mice	Immunological Features
IL-2	IL-2, IL-2Rα	Normal T and B and NK cell development; defects in the function of T-regulatory cells
IL-4	Il-4	Normal T and B cell development; impaired Th-2 type responses
IL-7	IL-7, IL-7Rα	Reduction in the number of thymocytes, bone marrow B cell precursors, and peripheral B, CD4 and CD8 T cells
IL-9	IL-9	Normal development of lymphoid compartment. Increased mast cell proliferation
IL-15	IL-15, IL 15Rα	Reduction of number of NK cells, intestinal intraepithelial lymphocytes (IELs), memory CD8 T cells.
IL-21	IL-21Rα	Normal number of NK, T and B cells. Decreased levels of IgG1

to hypothesize that the GH-R signaling also involved γc. Taking advantage of this observation, our group extensively studied the potential role of γc in GH-R signaling using BCLs from healthy control and γc-negative SCID-X1 patients. In particular, we demonstrated that various GH-induced events were impaired in γc-negative BCLs. First, while GH enhanced proliferation of control BCLs in a dose-dependent fashion, the functional response to GH of the BCLs of γc-negative patients was severely impaired despite a comparable cellular expression of GH-R molecules. Furthermore, the signal transduction properties of GH-R following GH stimulation in SCID patients was abnormal as compared to control BCLs and, in particular, in the pattern of protein tyrosine phosphorylation. In fact, in contrast to what observed in control BCLs, in patients cells GH stimulation failed to induce phosphorylation of STAT5 molecule [8]. This blockage in GH-R signaling was specific of STAT5 in that other molecules, as Janus kinase (JAK) 2, STAT1, extracellular signal-regulated kinase (ERKs) and STAT3 were normally phosphorylated following GH stimulation of B cells, thus suggesting the presence of pathways independently regulated. In support of this, transduction of patients' cells with the wild type γc gene corrected the functional and biochemical abnormalities, resulting in an appropriate nuclear translocation of STAT5 after GH stimulation. These data suggest that the γc is an important subunit of the GH-R signaling complex in BCLs, where it is required for STAT5 phosphorylation and nuclear translocation, and not for the activation of other molecules of GH-R signaling apparatus (Fig. **2**).

It is to note, that the immunological reconstitution of SCID-X1 patient through bone marrow transplantation paralleled the restoring of GH-R functionality, which resulted in a normal production of Insuline Growth Factor I (IGF-I) [24]. This would also imply that haematopoietic-derived cells represent an important source of those intermediate molecules that play a role in the GH-R functionality.

Whether the involvement of γc to the GH-R apparatus confers some additional properties to this receptor in haematopoietic cells and the specific functions in immune cells remain to be elucidated. In $CD34^+$ progenitors, γc participates in haematopoietic cell differentiation by interacting with Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) receptor-β (GM-CSFRβ) [25]. This interaction does not occur in normal NK cells or non-haematopoietic cells. Hence, the complexity of receptor signaling relies not only on the possibility that individual receptors interact one with each other, but also on a differential array of distinct subunits that may represent a hallmark of that receptor apparatus in a specific cell type.

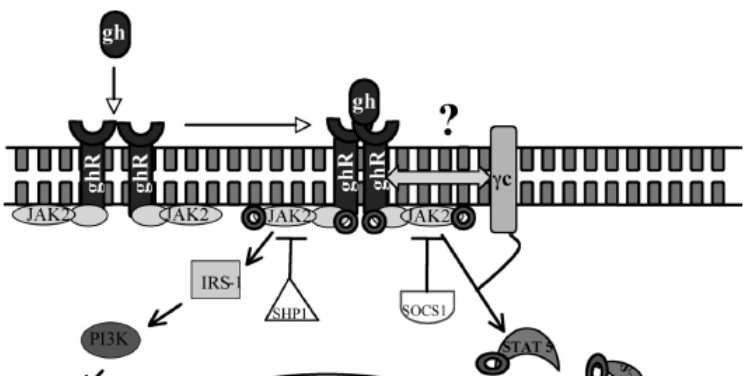
4. GROWTH HORMONE RECEPTOR SIGNALING

4.1. Signal Transduction Through GH Receptor

Endocrine and immune systems participate to an integrated network of soluble mediators that communicate and coordinate responsive cells to achieve effective functions in an appropriate fashion.

Cytokines and growth factors transmit signals through specific cell-surface receptors to the nucleus by activating intracytoplasmic signaling molecules. This process ultimately results in the activation of transcription factors.

GH is an important regulator of somatic growth, cellular metabolism, fertility and immune function. More than 400 functions linked to GH are long recognized. These are mediated by an array of distinct signals triggered by an individual receptor, thus implying



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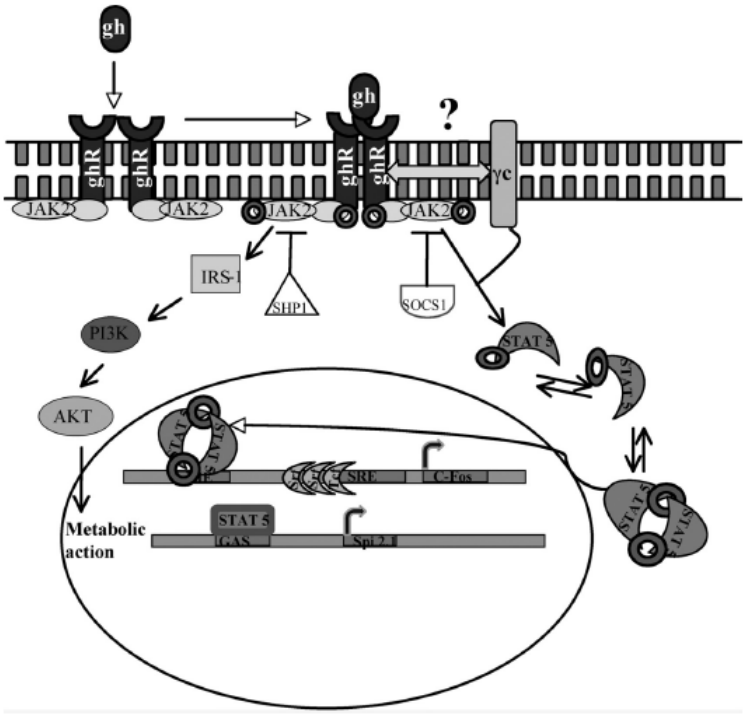

Fig. (2). Signaling pathway oof GH-R: a role for γc.

that diverse signaling pathways may be activated separately and in the context of a function-specific coordinating network [26]. The GH-R was the first member of the cytokine receptor superfamily to be cloned [27]. It consists of a transmembrane protein and an extracellular domain that contains six cysteines linked by disulfide bonds and one free cysteine [28]. Similarly to other members of the cytokine/hematopoietin receptor superfamily, the intracellular region contains two motifs. A proline rich motif, referred to as box 1, is situated in the proximity of the membrane and consists of eight amino acids. The other cytoplasmic motif (referred to as box 2) mainly consists of hydrophobic amino acids along with acidic amino acids. Like other members of the family, GH-R lacks intrinsic kinase activity and signal transduction is mediated by receptor associated cytoplasmic tyrosine kinases. A prominent role is played by the JAK2 that associates to the box 1 in the proximity of the GH-R cytoplasmic domain [29]. JAK2 belongs to the Janus family of cytoplasmic tyrosine kinases that includes JAK1, JAK2, JAK3, and tyrosine kinase 2 (tyk2). JAK2 activation is initiated by GH-induced receptor dimerization/oligomerization, which induces conformational changes resulting in JAK2 transphosphorylation and activation. The central role of JAK2 in this pathway is supported by the studies in which mutated GH-Rs fail to bind or activate JAK2, as clearly shown in several cell lines expressing truncated or mutated receptors [29,30]. These mutants fail to elicit GH dependent tyrosine phosphorylation of Src homologous and collagene-like (SHC) proteins, mitogen-activated protein kinases (MAPK), also designated as extracellular signal regulated kinases. After phosphorylation of JAK2, the receptor itself and several intracytoplasmic molecules are promptly phosphorylated on key tyrosine residues. Further signaling proteins recruited to JAK2/GH-R complex and/or activated in response to GH include: (1) SHC proteins that presumably lead to the activation of Ras/MAP (Rat sarcoma viral oncogene homolog/ Mitogen Activated Protein) kinase pathway; (2) insulin receptor substrates that have been implicated in the activation of phosphatidylinositol-3-kinase and the kinase AKT/PKB (Akt-8 retroviral oncogene/Protein kinase B); (3) phospholipases that lead to formation of diacylglycerol and activation of protein kinase C; and (4) a variety of proteins that are involved in the regulation of the cytoskeleton, including focal adhesion kinase, paxillin, tensin, CrkII, c-Src, c-Fyn, c-Cbl and Nck. GH is able to activate NF-κB pathways, leading to the expression of cell cycle mediators [31]. This process ultimately results in the activation of multiple transcription factors as STAT1, STAT3, STAT5a, and STAT5b, as detailed in the next paragraph. The tyrosine-phosphorylated STAT proteins dimerize and translocate into the nucleus, where they bind to specific DNA responsive elements of GH target genes, eventually inducing the activation of gene transcript [32].

The duration of GH-activated signals is a key factor in relation to the biological actions of the hormone. Removal of cell surface GH-Rs by endocytosis is an early step in the termination of GH-dependent signaling. Furthermore, suppressors of cytokine signaling proteins (SOCS) act as negative regulators of the main cytokine-activated signaling pathway, the JAK/STAT signal cascade. However, since the activation of GH-dependent signaling pathways is mainly based on protein phosphorylation on tyrosine, serine, or threonine residues, the obvious mechanism for deactivation of this process is the recruitment of a protein tyrosine phosphatase to GH-R/JAK2 signaling complex [33]. This phosphatase would dephosphorylate GH-R, JAK2, or the STATs themselves leading to signaling down-regulation. Several studies documented that there are at least three different phosphatases involved in the specific down-regulation of GH-R signaling: 1) SH2 domain-containing protein-tyrosine phosphatase 1 (SHP1 also known as PTP-1); 2) PTP1b; and 3) PTP-H1 [34]. Taking into consideration that a so huge number of GH actions are direct, a deep understanding of the regulatory mechanisms that control in an integrated fashion, presumably, distinct signaling pathways related to the same receptor through activation/down-regulation processes is mandatory. This knowledge would help understand tissue specificity of GH action and would allow to devise strategies to enhance individual functions of GH.

Thus, pharmacological targeting of specific negative regulators of GH signaling would have a remarkable potential to enhance or inhibit the beneficial effects of GH [33].

4.2. STAT Family

STATs factors represent a family of cytoplasmic proteins that participate in gene control in response to cell stimulation with various extracellular polypeptides [34]. So far, seven mammalian STATs: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 have been characterized [34]. These transcription factors mediate several functions by regulating the expression of effector genes involved in cell differentiation, survival, and apoptosis. All the STAT family members share in their structural arrangement functional motifs, and, in particular, the amino terminus that has a role in STAT dimerization. In addition, other motifs are a coiled-coil domain, involved in interactions with other proteins, a central DNA-binding domain, a SRC homology 2 (SH2) domain, a conserved single tyrosine residue that is phosphorylated following activation, and a carboxyl terminus that facilitates transcriptional activation.

In the cell biology, STAT proteins are essential regulators of cell proliferation, differentiation and survival in different cellular contexts, thus revealing their critical role in malignant transformation. Dysregulated STAT activation leads to increased angiogenesis and enhanced survival of malignant cells [35]. Knockout studies have also highlighted the function of STAT proteins in the development and function of the immune system and of their roles in maintaining peripheral immune tolerance and tumour surveillance.

Recent studies have shown that JAK–STAT signaling can be regulated through distinct mechanisms. Studies on STATs 1, 3 and 5 have elegantly demonstrated that unphosphorylated STATs shuttle between the cytoplasm and the nucleus in the absence of cytokine activation. Differently, the phosphorylated STATs are retained in the nucleus and are only released upon dephosphorylation through nuclear phosphatases [32]. STATs are activated by a number of cytokines, including interferons and interleukins, as well as growth factors and hormones. STAT1 is inducible by IFN -α/β and IFN-c, and is involved in anti-viral and anti-bacterial response, in growth inhibition, apoptosis, and tumor suppression [34]. STAT1 mediates the anti-viral and immune/inflammatory effects of IFNs through the induction of immune effector and inflammatory genes, such as major histocompatibility complex (MHC), costimulatory molecules, chemokines, complement, inducible nitric oxide synthase, and Fcγ receptor I (FcγRI) genes. STAT1 is also important in host anti-tumor responses and is also involved in non-immune functions, such as the regulation of bone formation and destruction. STAT3 is mainly activated by cytokines and growth factors, including IL-6 family members and epidermal growth factor (EGF), and is involved in mitogenesis, survival, anti-apoptosis, and oncogenesis. STAT3 is also required for optimal IL-2-induced T-cell proliferative responses by up-regulating the expression of IL-2Rα, a component of the high-affinity IL-2 receptor [36]. STAT3 is required for early development, as STAT3 null alterations in mice were embryonically lethal. Subsequent tissue-specific gene targeting showed STAT3 functions in wound healing in keratinocytes, regeneration of liver, mammary involution, and survival of many cell types [36]. Recently, tissue-specific deletion of STAT3 in bronchiolar and alveolar epithelial cells resulted in enhanced apoptosis in these cells following adenoviral infection, suggesting a cytoprotective function of STATs. STAT4 is predominantly stimulated by IL-12 and is involved in Th1 (T helper 1) development in humans. STAT4 is activated by IL-23 in murine cells as well, and, additionally, by IFN-α in human cells, being recruited to type I IFN

receptor through interaction with STAT2 [34]. As it may be expected, STAT4-deficient mice exhibit an impaired Th1 differentiation, IFN-γ production, and cell-mediated immune responses. STAT6 molecule is activated by IL-4 and participates in Th2 (T helper 2) development [35]. STAT5A and STAT5B are involved in prolactin and growth hormone signaling, respectively.

Multiple inhibitory mechanisms down-regulate cytokine-JAK–STAT signaling. Down-regulation of this pathway is likely to be important for homeostasis and the prevention of chronic inflammation or autoimmunity. Both constitutive inhibitory pathways and inducible mechanisms have been described. These mechanisms can act at several levels in cytokine signaling, targeting or the receptors, or JAK proteins, or the STATs themselves. Constitutive inhibitory mechanisms include proteolysis, dephosphorylation, and interaction with inhibitory molecules termed Protein Inhibitors of Activated STATs (PIAS). Three major regulated or inducible inhibitory mechanisms have been identified, mediated by down-regulation of receptor expression, through the induction of inhibitory molecules termed SOCS proteins, and by rapid MAPK or protein kinase C (PKC)-dependent modification of pre-existing signaling components. One proposed mechanism for degrading cytokine signaling components, in which STATs are mostly involved, is the coupling of signaling proteins to proteasomes by SOCS molecules. SOCS family of proteins consists of eight members. These SOCS proteins are generally expressed at low levels in unstimulated cells and become rapidly induced by cytokines, thereby inhibiting JAK–STAT signaling and forming a classic negative-feedback loop [33]. Finally, the modulation of JAKs and STATs by various protein modifications and the cross-talk between different JAK–STAT pathways and other cellular signaling pathways provide additional levels of regulation of cellular homeostasis [33].

A potential novel pharmacological strategy may be to develop specific drugs that can specifically target the JAK-STAT regulators or the motifs implicated in such intermolecular interactions.

Once GH-R signaling is activated, GH participates to an integrated network with other mitogenic factors, as hepatocyte growth factor in liver cells, basic fibroblast growth factor in cartilage, epidermal growth factor in kidney, estrogen receptors in the uterus, bone morphogenetic proteins in various tissues, all of them being involved in tissue growth.

The effects of GH on growth are mostly mediated by intermediate factors, of which the IGF-I is the most studied [37]. GH upon binding to its cognate receptor initiates the signaling cascade, which culminates in the regulation of multiple genes, including IGF-I and its major binding protein the IGF binding protein-3 (IGFBP-3). The binding protein and the acid labile subunit (ALS) prolong the half-live of IGF-I and regulates its tissue distribution and bioavailability at the cellular level [38]. The linear growth is a complex process. GH and IGF-I participate as combined factors or separately to this process. [39]. The double KO IGF-I + GH-R mouse revealed that 35% of body growth was due to IGF-I alone, 14% to GH alone and 34% to a combined effect of GH and IGF-I. [40]. Despite the close relationship between GH and IGF-I, GH appears to have many cellular effects that are independent of IGF-I, and are most probably direct effects of GH mediated by its cognate receptor [39]. Moreover, all tissues but the liver express IGF-I transcripts in the absence of a functional GH-R, thus indicating that the molecule has additional biologic effects that are GH independent. [39].

In addition, evidence supports a role for GH acting as a cytokine in the immune system under conditions of stress, counteracting immunosuppression by glucocorticoids [31]. Lymphoid cells express the GH-R and GH can be produced by immune tissues, suggesting an autocrine/paracrine mode of action of GH. Moreover, GH can, directly or indirectly by the production of IGF-I, promote cell cycle progression and prevent apoptosis of lymphoid cells as well as a wide variety of other cells.

A so huge number of distinct biologic functions of the GH/IGF-I axis may be expressed through the existence of many separately regulated signaling pathways, as above described. Baixeras *et al.* have recently demonstrated that both GH and IGF-I are able to promote cell survival and proliferation through independent and different pathways, thus indicating a potential function related specificity of an individual pathway [41]. The effects on the proliferation were shown to be mainly mediated by the PI-3 kinase (Phosphatidylinositol-3-kinase) /Akt pathway and the transcription factor NF-kB. Moreover, GH regulates the expression of several cell cycle mediators, such as Bcl-2, cMyc and cyclin proteins [31], The transcription factor NK-kB is involved in the regulation of many functions of the immune system, and its activation by GH would be one of the keys explaining the cytokine-like effects of GH on the immune system. Since NF-kB is related to the transduction of survival signals by GH, Jeay *et al.* suggested that GH treatment may partially protect immune cells against apoptosis induced by stress conditions and deregulated expression of GH may participate to the development of malignancies of immune cells, such as leukemias or lymphomas [31].

In addition to GH, also IGF-I, in turn, activates specific cellular pathways, through tyrosine kinase activation and phosphorylation, leading to the various biological actions. IGF-I exerts many biologic effects, as induction of cell growth through the activation of cell cycle machinery, maintenance of cell survival by acting on the Bcl family members, and induction of cellular differentiation through poorly characterized mechanisms [42]. Overall, IGF-I inhibits apoptosis as well, thus acting as cell survival factors [38]. The antiapoptotic function of IGF-I is mediated by IGF-I receptor (IGF-I-R). Many studies have clearly illustrated that IGFBPs can affect cell cycle progression and apoptosis by preventing IGF-I-R activation [38]. Thus, IGFBP-3 can also modulate the antiapoptotic effect of IGF-I by regulating the IGF-I/IGF-I-R interaction. Modulating IGFBPs by proteolysis also plays a role in regulating their proliferative effect. There is also evidence that IGFBP-3 can induce apoptosis on its own [38,43].

The liver is thought to be the major source of IGF-1. Liver deficient IGF-I (LID) mice show a major (75%) reduction in circulating IGF-I levels, whereas the expression in non-hepatic tissues was normal [44]. However, these mice are similar to wild-type mice with respect to body weight, body length or femoral length, thus indicating that the normal growth is mediated by autocrine/paracrine actions of IGF-I combined with non-hepatic sources of circulating IGF-I [44]. In humans, we documented that stem cell transplantation, performed to treat a severe combined immunodeficiency associated with a remarkable peripheral hyporesponsiveness to GH, was capable to restore IGF-I levels to normal values, thus adding in favour of a lymphocyte source of the mediator [24].

5. COMPLEX CLINICAL PHENOTYPES AFFECTING BOTH IMMUNE AND ENDOCRINE SYSTEMS

5.1. Murine Models

The Murine Model of SCID-X1

KO mice for the γc of the IL-2R represent the human counterpart of the SCID-X1 phenotype. These animal models show significant reduction of T, B, and NK cells (Di Santo *et al.*,1995;Cao XG *et al.*,1995) associated with normal thymic architecture but reduced thymic cellularity. Thymic cellularity is controlled by γc-dependent cytokines at the level of pro- and pre-T-cell development. In particular, each stage during pro- and pre-T-cell development involves γc cytokines for a proper T- lymphocyte production. Among T-cell developmental stages, the transition from stage 2 ($CD44^+CD25^+$) to stage 3 ($CD44^-CD25^+$) pro-T-cell, seems to be the critical stage for γc. In fact, in γc-deficient mice a higher number of pro-T cells is observed. Moreover, the few thymocytes in γc-deficient mice exhibit a relatively normal pattern of CD4 and CD8 expression (Tho-

mas R. *et al.*, 1999). However, an elevated number of $CD4^+$ T cells is seen in periphery differently from $CD8^+$ T cells that are expressed at low level. This observation suggests that γc could be implicated in the regulation of thymic export and/or in the homeostasis of the peripheral T, as well.

Even if human and mice models of SCID-X1 are similar in the decrease of T and NK cells, there is marked difference in B-cell development. In fact, differently from what observed in the animal model, human SCID-X1 shows normal or high level of B cells, as also observed in IL- 7 deficient mice. However, in spite of such differences, SCID-X1 mice represent an important model to better understand the pathophysiology of the human disease.

The Murine Model of STAT 5 Knock Out

Most of the current knowledge about the biological function of STAT family members has been achieved through disruption approaches and studies of KO mice [45]. In particular, all seven mouse STAT genes have been disrupted in transgenic animals [46]. Most of these animals have been studied and the results obtained have reinforced the notion that STAT proteins are a critical point of cytokine signaling specificity [47]. Unlike most of the STATs, the two STAT5 proteins are activated in reply to a variety of cytokines as well as tyrosine kinase receptors. Then, it was supposed that STAT5a/b would have very fundamental functions in regulating cell growth.

Differently from the other STATs, the study of the STAT5 function *in vivo* has been more difficult because there are 2 isoforms, STAT5a and STAT5b, very close on the chromosome 11 [7]. This position made quite difficult to create a double KO. In particular, these two proteins are 96% related at the amino acid level, but diverge, principally, at the carboxyl terminus. However, the diversities led to different DNA-binding specificities related to a single amino acid variation in the DNA binding field. Nevertheless, these two proteins control the expression of common as well as distinct genes. Moreover, slight difference in the tissue allocation of their mRNA has been found, thus contributing to their functional specificity. At first, STAT5a and STAT5b KO were individually produced and examined.

STAT5a gene targeting in mice confirmed its principal role in mammopoietic and lactogenic signaling [46]. In fact, the phenotype of STAT5a deficient mice showed impaired mammary gland development and lactogenesis. STAT5a KO mice developed normally and were indistinguishable from hemizygous and wild-type littermates in size, weight and fertility [47]. Unlikely STAT5a KO, STAT5b KO mice have a most important failure of several responses associated with growth hormone secretion. In particular, the deletion of STAT5b alone gives a phenotype analogous to that observed in growth hormone receptor deficient mice, predominantly resulting in failure of postnatal growth development. STAT5b mediates the sexual dimorphic effects of GH pulses in liver and other target tissues. This comes out from the observation that in STAT5b KO males body growth rates and male-specific liver gene expression were decreased to the levels observed in wild-type females. On the contrary, female-predominant liver gene products were increased to a level intermediate to those of wild-type male and female levels. A recent study, analyzing mice which lacked STAT5a and/or STAT5b gene, confirmed these findings [48]. In addition, STAT5a/b double KO mice were found to have a defect in the development of functional corporal lutea in the ovary, resulting in female infertility. Female infertility is not observed in the STAT5a or STAT5b KO mice alone, demonstrating the functional redundancy of the STAT5 proteins [46].

The role of STAT5 in the immune system was also extensively analyzed. Splenocytes from STAT5a KO mice have a partial defect in anti-CD3-induced proliferation that can be overcome by high doses of IL-2 [49]. This is because STAT5a activation is responsible for IL-2-induced IL-2Rα expression in T lymphocytes. Although IL-2Rα expression is also defective in STAT5b KO mice, it cannot be corrected by administration of high doses of IL-2. Splenocytes from STAT5b KO mice exhibited greatly diminished proliferation in response to IL-2 and IL-15. Basal as well as IL-2- and IL-15-mediated augmentation of NK cytolytic activity was also greatly diminished in STAT5b KO mice. The percentage of NK cells expressing IL-2Rβ as well as the levels of IL-2- and IL-15-induced perforin expression in splenocytes were also significantly diminished in STAT5b KO mice. These data indicate that STAT5b is essential for potent NK cell-mediated proliferation and cytolytic activity. In addition, bone marrow-derived macrophages from STAT5a KO mice have a defect in GM-CSF-induced proliferation as well as in the expression of GM-CSF-dependent genes such as CIS and a bcl-2 like gene A1 [50]. One of the surprising results from disruption studies of either STAT5a or STAT5b was the observation that in either cases the development of the haematopoietic system took place apparently normally, in spite of the many studies showing STAT5 activation in response to several haematopoietic cytokines as erythropoietin, IL-2, IL-3, IL-5, GM-CSF, IL-7, and CSF-1. Of note, a few of these cytokine are implicated in haematopoietic development and differentiation. However, an impaired proliferation of peripheral T lymphocytes was observed in STAT 5 a/b KO mice, even if this characteristic is due, probably, to a defect in the cell cycle entry rather than to a decreasing of IL-2 receptor expression. In fact, while lymphopoiesis was normal, T cells from double KO mice showed a marked failure to undergo cell cycle progression and a diminished expression of proteins fundamental for proliferation [47].

Thus, it is clear how STAT5 proteins are strongly correlated with some oncogenic events, such as proliferation and apoptosis [47]; so, the therapeutic inhibition of these transcription factors may be proven helpful for those diseases characterized by an alteration of cellular homeostasis.**5.1. Human Models**

The Human Model of SCID-X1

Primary immunodeficiency syndromes represent a group of distinct genetic disorders inherited in an autosomal recessive or X-linked mode. It has become evident that most of the immunodeficiencies are disorders of the intra- or inter-cellular communication network. The most common form of SCID accounting for approximately half of all cases, with an estimated incidence of 1:150000 to 1:200000 live births, is the SCID-X1 [51,52]. This form of SCID is characterized by the absence of mature T and NK lymphocytes and absent immunoglobulin synthesis, despite the presence of a normal or sometime elevated number of B cells. In SCID-X1 patients, T cells are absent not only in the peripheral blood but also in the central and peripheral lymphoid organs suggesting an early block in the T-cell differentiation pathway [53]. Furthermore, although peripheral B-cells exhibit a normal phenotype, SCID-X1 patients B cells are not functional, even after T-cell reconstitution by means of bone marrow transplantation [54,55]. Mutations of the γc gene are responsible for the SCID-X1 [56]. As above mentioned, the SCID-X1 is also associated with GH hyporesponsiveness that results in the impairment of linear growth [7].

The Human Model of STAT5 Mutations

STAT5b is involved in GH mediated IGF-I gene transcription and production of IGF-I and in transcription and production of IGFBP-3 and the Acide Labile Subunit (ALS) as well [57]. This came out from the observation of patients carrying mutations of STAT5b gene. To date, a total of six patients carrying genetic abnormalities of STAT5b have been identified [2,4,5]. Interestingly, five of the six cases are female, indicating that in humans, unlike mice, STAT5b is crucial in the growth of both males and females. Serum concentrations of IGF-I, IGFBP-3 and ALS were strikingly reduced, while basal and stimulated GH concentrations were either

normal or increased. The clinical phenotype of these patients was characterized by growth failure and immunodeficiency. Recurrent pulmonary infections, chronic diarrhoea, severe eczema, herpes keratitis, severe varicella, juvenile arthritis, lymphoid interstitial pneumonia with fibrosis, were reported in these patients [1-5]. Several immune deficiencies, such as decreased number and function of $CD4^+CD25^+$ regulatory T cells, reduced up-regulation of CD25 and of the common γc cytokine receptor and perforin in response to IL-2 were documented, thus suggesting that STAT5b propagates an important IL-2-mediated signal for the *in vivo* accumulation of functional regulatory T cells [42]. However, the relationship between endocrine and immune dysfunctions in patients with STAT5b alterations are not yet completely defined, in that one male patient with a homozygous mutation of STAT5b with severe short stature did not suffer from any infection and did not show any immunological abnormality [58]. Of note, this patient was the only male described with STAT5b deficiency, thus suggesting the possibility that the phenotype is sex-dependent.

On the contrary, no immunodeficiency was observed in patients with low IGF-I due to GH-R mutation, IGF-I gene alterations [59,60], or mutations in the IGF-I-R gene [61]. This finding would mean that a number of distinct pathways under separate regulatory mechanisms cooperate in an integrated fashion to achieve a specific function.

As a matter of fact, gene disruption of additional signaling molecules implicated in GH-R signaling does not lead to a combined endocrinological and immunological disorder, thus implying that the integration of distinct pathways are responsible for a full expression of a certain function. For istance, targeted disruption of ERK1 in rodent models results in mice that are of normal size but defective in thymocyte maturation [1]. In keeping with this, in humans normal GH-induced ERK signaling seems to be insufficient to compensate the reduced IGF-I expression due to absence of STAT5b or GH-R mutations [2,62]. Certainly, STAT5b seems a shared component between signaling pathways implicated in both immunological and endocrine functions. Many cytokines, as IL-2, IL-7, IL-21 and IFN-γ, can activate STAT5b. Hwa *et al.* have recently demonstrated the cytokine IFN-γ, like GH, could not activate mutant STAT5b, resulting in a markedly reduced expression of the IGF-I gene [3]. It is, therefore, likely that cytokines, important for cellular immunity such as IL-2 and IFN-γ, require STAT5b for efficient regulation of multiple genes, also including IGF-I [3].

The Human Model of NF-κB Mutation

As a further example of complex disease involving both endocrine and immune system, it has been recently documented that a heterozygous mutation of the Inhibitor of NF-κB (I-κBα) gene was implicated in a clinical phenotype characterized by severe immunodeficiency and clinical signs of partial GH Insensitivity (GHI) [6]. The NF-κB signaling pathway plays a crucial role in many physiological process, such as the innate and adaptative immune responses, apoptosis and inflammation [63]. NF-κB is bound to I-κB and his activation involves the phosphorylation of the inhibitor, which results in I-κB degradation and releasing of NF-κB. Free NF-κB translocates into the nucleus and activates transcription of target genes.

6. GH/IGF-I AXIS AND RISK OF CANCER

As for the relationship between the GH/IGF-I axis and the risk of developing cancer, no conclusive data are available. The risk of developing cancer is determined by a combination of genetic factors and environmental effects, in particular diet and lifestyle. There is increasing evidence that the GH/IGF-I axis may provide a link between these factors and the development of cancers through the regulation of normal cell proliferation, differentiation and apoptosis [64].

Recently, several epidemiological reports have been systematically reviewed to define the association between circulating IGF-I and IGFBP-3 concentrations and the risk of developing cancer [65]. Elevated serum levels of IGF-I have been associated with an increased risk of breast, prostate and colorectal cancer in humans in several epidemiological studies [66,67]. In the transformed cell, there are abundant data showing that IGF-I-R regulates cancer cell proliferation, survival and metastasis [68]. Targeted disruption of the IGF-I-R results in almost complete resistance of mouse embryonic fibroblasts to transformation, while inhibition of the IGF-I-R almost completely blocks colony formation by melanoma cells in soft agar and substantially reduces tumour formation in a mouse xenograft model [66]. IGF-I is mitogenic and exerts an important antiapoptotic effect, whereas IGFBP-3, which is thought to inhibit growth through ligand sequestration, is supposed to also have antiproliferative and proapoptotic effects, thus interfering with tumor growth [65]. However, the association between elevated concentrations of IGF-I and the increased risk of cancer is modest and varies between different sites.

On the opposite site, several studies have documented that other cancer risk factors, including diet and lifestyle factors, have an effect on IGF-I and IGFBP-3 concentrations [65].

Differently from IGF-I and IGFBP3, the involvement of GH in the physiopathology of cancer is still questionable. As matter of fact, GH treatment positively influences in parallel both IGF-I and IGFBP-3, contributing to the maintenance of the balance between these two molecules. GH, through its receptor-linked signal transduction mechanism, stimulates the expression of several genes whose significance in the development of cancer is still unknown. Overall cancer incidence is not increased in acromegaly.

STAT molecules and, in particular, STAT3 and STAT5, have been demonstrated to directly participate in tumor development and progression [69,70]. STATs participate in oncogenesis through up-regulation of genes encoding apoptosis inhibitors and cell cycle regulators such as Bcl-x_L, Mcl-1, cyclins D1/D2, and c-Myc. Moreover, tumor cells possessing activated STAT3 or STAT5 are predicted to be resistant to chemotherapeutic agents that may utilize similar apoptotic pathways pathway. It has been clearly documented that inhibition of constitutively active STATs result in growth inhibition and induction of apoptosis in tumor cells [70,71].

7. THERAPEUTIC PERSPECTIVES

As a matter of fact, the expression of more than 400 biologic effects functionally related to an individual receptor-ligand, GH/GH-R, may occur based on the assumption that an array of distinct pathways may be integrated in a tightly coordinated fashion. A primary aim of novel therapies should be to increase our understanding of the relationship between inhibitory and stimulatory actions of GH-related signaling pathways. The GH-R signaling apparatus also involves potent mitogenic molecules such as γc and STATs that play a role in the cell proliferation and, in general, in cell homeostasis. Thus, in principle it is conceivable to hypothesize a pharmacological intervention in tissue engineering and remodelling by interfering in such pathways. The dissection of individual signaling pathways and the identification of the specific molecule implicated in that certain function remain the major goal of such novel therapeutic approaches.

The development of targeted therapeutics to activate growth inhibition (e.g. cancer) or stimulation (e.g. tissue engineering) represents the major issue.

Progress in defining the pathogenic implications of IGF-I/IGF-I-R and downstream molecules in neoplasia might lead to the development of novel targeting strategies to fight those cancers that may be proven responsive. Drugs to disrupt IGF-I-R have been developed. Most of the anti-IGF-I-R strategies have been directed against the receptor itself. A different approach may be the removal

of the ligand, or the use of tyrosine kinase inhibitors. This class of reagents are good candidate for the treatment of a number of different cancer types. However, because of the ubiquitous nature of IGF-I-R expression and action, blockade of IGF-I-R could affect multiple tissues. Therefore, careful attention to future clinical trials of these therapeutic targeting in combination with chemotherapy will be necessary [72].

The most potent therapy for reducing serum IGF-I levels is Pegvisomat, the GH-R antagonist. The potential role of this compound in the treatment of IGF-I-influenced cancers is intriguing. In animal models of metastatic colon cancer, Pegvisomat in combination with conventional chemotherapy virtually abolishes the metastatic disease [64]. It has also been reported to exert inhibitory effects against breast cancer cell lines implanted into athymic mice [73].

As above discussed, aberrant STAT activation is also associated with oncogenesis. Thus, the development of selective inhibitors of STAT activation may be a promising area in the field of novel anticancer therapeutics [70]. However, whether this can be translated into the clinical setting and used for the treatment of human cancers remain to be extensively proved. In the near future the first goal of studies in this field will be the identification of the individual molecule implicated in that specific GH related function. This may lead to targeted therapy aimed at potentiating or abolishing only that biologic effect.

The participation of γc to several receptors implicated in the immune response would also suggest potential effect of GH in immune response, even though the precise role of the hormone is not completely defined. It should be noted that overexpression of γc in engineered lymphocytes, used for gene correction of SCID-X1 and mutation of γc resulted in neoplastic transformation in 3 cases. Even though this side effect was interpreted as due to insertional oncogenesis, it is also conceivable to hypothesize that overexpression of the molecule exerts an oncogenic effect *per se*.

ABBREVIATIONS

AKT	=	Akt-8 retroviral oncogene
ALS	=	Acide Labile Subunit
BCLs	=	B cell lines
EGF	=	Epidermal Growth Factor
ERK	=	Extracellular signal-regulated kinase
FcγRI	=	Fcγ receptor I
γc	=	Gamma chain
GH	=	Growth Hormone
GHI	=	Growth Hormone Insensitivity
GH-R	=	Growth Hormone Receptor
GM-CSF Factor	=	Granulocyte Macrophage-Colony Stimulating
GM-CSFRβ Factor receptor-β	=	Granulocyte Macrophage-Colony Stimulating
IFN	=	Interferon
Ig	=	Immunoglobulin
IGFBP	=	Insuline Growth Factor Binding Protein
IGF-I	=	Insuline Growth Factor I
IGF-I-R	=	IGF-I receptor
JAK	=	Janus Kinase
IL	=	Interleukin
LID	=	Liver deficient IGF-I
MAPK	=	Mitogen Activated Protein Kinase
MHC	=	Major Histocompatibility Complex
NF-κB	=	Nuclear Factor-κB
NK	=	Natural Killer
PIAS	=	Protein Inhibitors of Activated STATs
PI-3 kinase	=	Phosphatidylinositol-3-kinase
PKB	=	Protein Kinase B
PKC	=	Protein Kinase C
PTP	=	Protein Tyrosine Phosphatase
Ras	=	Rat sarcoma viral oncogene homolog
SCID-X1	=	X-linked Severe Combined Immunodeficiency
SH2	=	SRC homology 2
SHC	=	Src homologous and collagene-like
SHP phatase	=	SH2 domain-containing protein-tyrosine phos-
SOCS	=	Suppressors of cytokine signaling
STAT tion	=	Signal Transducers and Activators of Transcrip-
TGF-β	=	Tumor Growth Factor- β
Th	=	T helper
TNF	=	Tumor Necrosis Factor
γc	=	γ chain
TCR	=	T Cell Receptor

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CONCLUSIVE REMARKS

The rapid expansion in the past two decades in the understanding of the molecular basis of a large variety of novel congenital immunodeficiencies has provided valuable information on the signal transduction general mechanisms, that goes far beyond the comprehension of the individual disease. In most cases, the altered molecules are axclusively expressed in hematopoietic cells, while in other case they are not restricted to a certain cell type. This leads to kore complex clinical phenotypes, which contribute to unravel previously unappreciated non-hematopoietic functions of signaling proteins and the mechanism of coordination and integration of several pathways. Moreover, this knowledge will help define potential new therapeutic strategies through novel molecular targets, drive stem cell development into the desired differentiation pat and ameliorate our comprehension of tissue engineering.

CHAPTER 5
COMPLEX INTERACTIONS BETWEEN ENDOCRINE AND OTHER BIOLOGICAL SYSTEMS: THE MODEL OF HYPOTHYROIDISM

5.1 Introduction and aims

Thyroid hormones (TH) are key regulators of metabolism and development and are known to have pleiotropic effects in many different organs. TH act in practically all tissues of the body and influence enzyme concentration and activity, the metabolism of carbohydrates and lipids, vitamins and mineral salts, basal metabolism or calorigenesis; they also stimulate the consumption of oxygen and act in other endocrine systems (1). The influence of TH on growth is related to its activity in synthesis and degradation of proteins. Moreover TH are essential for the development of the central nervous system (2).

Clinical conditions resulting from deficiency in the production or in the activity of TH will depend on the degree and duration of the deficiency, and will affect basically all tissues to a lower or greater extent. Thyroxine is a hormone essential for the development and functioning of the brain, both in utero and in the postnatal period. Infact, TH play an important role in several neurobiological mechanisms: neurogenesis and neuronal migration, formation of axons, synaptogenesis and regulation of neurotransmitters. The action of thyroxine in the brain, occurs through interaction with a specific hormone receptor. Recent studies have demonstrated the existence of 4 different isoforms of this receptor that is expressed during brain development, at different times, regions such as caudate, hippocampus and cortex, regions involved in cognitive activities such as attention and memory, but also the cochlea and retina, with important implications on the processes auditory and visual (3). This shows the extreme importance of thyroxine on the maturation of the brain and the damage that may result from its absence during critical periods of development in children with congenital hypothyroidism. Deficiency of TH during fetal and newborn life extends tissue immaturity, leads to hypoplasia of cortical neurons, delayed myelinization and reduced vascularization. If hormone replacement therapy is not carried out soon after birth, lesions will become irreversible, and the child's neuropsychomotor development will be damaged, leading mental retardation. Infact, Congenital Hypothyroidism (CH) is the commonest cause of preventable mental retardation, with an incidence in Italy of around

one in three thousand live births (**4**). However, there are very few data on long-term effect of L-thyroxine treatment on the organs and systems target of TH. In this regard, long-term L-T4 therapy in patients with CH is not generally associated with side effects at replacement dosages, however, previous studies have reported common episodes of L-T4 overtreatment and undertreatment in patients with CH, which are commonly attributed to both the need to maintain serum TSH levels within normal range even though this requires increased free T4 (FT4) concentration and to the patients' inadequate compliance (5, 6).

Beyond their role on somatic growth and mental development, thyroid hormones exerts other important effects on many other organs and tissues. Recent data suggest that the cardiovascular system is a major target of thyroid hormone action (7) and a wide spectrum of cardiovascular changes has long been recognized in adults with overt thyroid dysfunction (7-9) and, more recently, in subclinical thyroid dysfunction (7, 10,11). In this regard, patients with CH receiving long-term LT-4 replacement therapy may present a subset of patients at risk of subclinical dysthyroidism. However, long-term cardiac function has never been investigated in children and adolescence with congenital hypothyroidism detected by neonatal screening and treated from the first month life. Few and conflicting data, in fact, have been reported only in neonates with congenital hypothyroidism.

As described above, clinical conditions resulting from deficiency in the production or in the activity of TH will depend on the degree and duration of the deficiency. However, consequences of milder thyroid disfunction are less well characterized than those in overt hypothyroidism.

Suclinical hypothyroidism (SH) represents a condition of mild to moderate thyroid failure characterized by normal serum levels of thyroid hormones with mildly elevated serum TSH levels (12). The prevalence of SH has been reported to be between 4 and 10% of adult population samples (12). In adults, progression from mild to overt hypothyroidism may be related to the cause of thyroid hormone deficiency, the basal TSH value and the patient's age. Moreover SH may be persistent or transient **(12)**. The natural course of SH in aged patients has been reported to be characterized by frequent normalization of TSH elevation, whereas the risk of progression to overt hypothyroidism was significantly greater in the ones with high TSH concentrations at baseline (13). The global prevalence of symptoms in patients with SH remains controversial. Recent data suggest that adult with subclinical hypothyroidism may

present increased risk of cardiovascular morbidity (14, 15), impaired lipid profile , increased inflammatory markers and alterations in coagulation parameters (12), even if results are still contrasting.

In childhood and adolescence Subclinical Hypothyroidism is less frequent than adults (16). Data concerning the natural evolution of subclinical hypothyroidism in childhood and adolescence are very scanty. All the avalaible reports on the spontaneous evolution of SH in both aged and young patients have been based, up to now, on unselected populations including patients with either thyroid disorders or other pathological causes that are known to be able to affect SH development and evolution. (13, 16).

Therefore, the aim of this phase of the project are the followings:

- Evaluate whether long-term LT4 replacement therapy in young adults with CH is associated with cardiovascular abnormalities. To this aim cardiac function, exercise capacity, intima-media thickness and endothelial function were evaluated in young adults with congenital hypothyroidism, compared with healthy controls.
- To evaluate, through a multicenter study, the natural course of subclinical hypothyroidism in children and adolescents with no chronic diseases and no risk factors that interfere with the progression of subclinical hypothyroidism.

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5.2 LONG-TERM CARDIOVASCULAR EFFECTS OF LEVOTHYROXINE THERAPY IN YOUNG ADULTS WITH CONGENITAL HYPOTHYROIDISM

ORIGINAL ARTICLE

Endocrine Care

Long-Term Cardiovascular Effects of Levothyroxine Therapy in Young Adults with Congenital Hypothyroidism

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Context: Congenital hypothyroidism (CH) is the most prevalent endocrine disorder in the newborn and is routinely treated with life-long levothyroxine replacement therapy. Although several studies have demonstrated that such therapy may impact on the cardiovascular system, little is known with regard to the effects of long-term levothyroxine administration in patients with CH.

Objective: The aim of the current study was to evaluate whether long-term levothyroxine replacement therapy in young adults with CH is associated with cardiovascular abnormalities.

Patients and Methods: Thirty young adults with CH aged 18.1 ± 0.2 yr and 30 age- and sex-matched controls underwent cardiac and carotid Doppler ultrasound and symptom-limited cardiopulmonary exercise testing. Hypothyroidism was diagnosed by neonatal screening, and levothyroxine treatment was initiated within the first month of life and carefully adjusted to maintain TSH levels in the normal range and free T_4 in the high-normal range.

Results: Compared with controls, hypothyroid patients exhibited left ventricular diastolic dysfunction, impaired exercise capacity, and increased intima-media thickness. At multiple regression analysis, the number of episodes of plasma TSH levels less than 0.5 mU/liter and greater than 4.0 mU/liter from the age of 1 yr onward, and mean TSH plasma levels during puberty were independent predictors of diastolic filling and cardiopulmonary performance indexes (multiple r values: 0.61–0.75).

Conclusions: Long-term levothyroxine treatment in young adults with congenital hypothyroidism is associated with impaired diastolic function and exercise capacity and increased intima-media thickness. (*J Clin Endocrinol Metab* 93: 2486–2491, 2008)

Congenital hypothyroidism (CH) is the most prevalent endocrine disorder in the newborn, with a rather constant worldwide incidence of permanent CH of 1:3000 to 1:4000 newborns (1, 2). Newborn screening programs have represented a major achievement insofar as early diagnosis and long-term treatment with levothyroxine (L-T4) have resulted in normal development in the vast majority of patients, with only up to 10% of patients affected by residual problems regarding mental development and neurological symptoms.

The cardiovascular system is very sensitive to thyroid hormones, and a wide spectrum of cardiovascular changes has long been recognized in overt and, more recently, in subclinical thyroid dysfunction (3–5). In this regard, patients with CH receiving long-term L-T4 replacement therapy may represent a subset of patients at risk of subclinical dysthyroidism. Indeed, previous studies have reported common episodes of L-T4 overtreatment and undertreatment in patients with CH, which are commonly attributed to both the need to maintain serum TSH levels within normal range even though this requires increased free T_4 (FT4) concentration and to the patients' inadequate compliance (6, 7).

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Abbreviations: A, Late diastolic flow velocity; BMI, body mass index; CH, congenital hypothyroidism; E, early diastolic flow velocity; FT4, free T_4; IMT, intima-media thickness; L-T4, levothyroxine; LV, left ventricular; mTSH, serum TSH; peak VO_2, highest VO_2 value measured; TDI, tissue Doppler imaging; VO_2, oxygen consumption.

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Therefore, the aim of the current study was to evaluate whether long-term L-T4 replacement therapy in young adults with CH is associated with cardiovascular abnormalities.

Patients and Methods

Patient population

Thirty-two adolescents (21 females,11 males) affected with CH aged 16.0–20.0 yr (18.1 ± 0.2 yr) participated in the study. All patients were detected by neonatal screening and followed longitudinally, from the time of diagnosis to the time of the study. The diagnosis was confirmed by serum thyroid function tests. L-T4 replacement therapy was started, immediately after the first evaluation, at a mean age of 26 ± 0.9 d (range 12–30 d) and at a mean initial dose of 6.4 ± 0.2 kg/d. Replacement therapy was modified during follow-up according to clinical and hormonal evaluation to maintain normal serum TSH and serum FT4 in the upper normal range (2, 8). The etiological diagnosis of CH was made on the basis of 99mTc-pertechnetate or iodine-123 thyroid scans at the time of diagnosis or the age of 3 yr, after the withdrawal of L-T4 therapy for 6 wk. Cases were classified into three groups: athyreosis (n = 7), ectopic (n = 18), and eutopic gland (n = 7). At study entry, all subjects had completed their pubertal development, and females had regular menstrual cycles. All patients had already reached their adult height (defined as a growth of less than 1.0 cm/yr during the preceding year) (8). Serum TSH and thyroid hormones and routine blood analysis were periodically assessed (every 6 months). At the time of cardiac testing, all subjects were euthyroid. Five patients were mild smokers (less than 10 cigarettes per day) and three were moderate drinkers (beer or wine occasionally). Previous or current cardiovascular, respiratory, renal, or other chronic diseases as well as obesity were exclusion criteria for entering the study.

Thirty-two healthy adolescents comparable for age, sex, body mass index (BMI), and smoking and alcohol use rates and physical activity, including recreational exercise, participated in the study as controls. All of subjects from the control group were from the same geographic region of patients with CH and were matched by their socioeconomic status. Informed consent was obtained by all patients or their parents (if the patient was under 18 yr of age), and the study was approved by the Ethics Committee of the Federico II University.

Study protocol

At study entry, all subjects underwent height, weight, heart rate, systolic and diastolic blood pressure measurement. The degree of adiposity was expressed as BMI. To evaluate the influence of thyroid hormone status on cardiac performance during puberty, mean values were calculated from all serum TSH (mTSH) and FT4 evaluation carried out from the onset of puberty to the time of the study. The mean L-T4 dose taken in the same period of time was also calculated (9). Based on the normal TSH range in our laboratory (0.5–4.0 mU/liter), we also calculated an index of overtreatment (number of episodes when serum TSH was < 0.5 mU/liter during follow-up from the age of 1 yr onward) and an index of undertreatment (number of episodes of serum TSH > 4.0 mU/liter). We also pooled all episodes of overtreatment and undertreatment as an index of patients' inadequate treatment. Measurements younger than 1 yr of age were excluded because TSH serum levels were still above the normal range in a vast majority of patients. To assess the degree of TSH derangement, *i.e.* the deviation from the range 0.5–4.0 mU/liter used, we calculated the average of all TSH values greater than 4.0 and less than 0.5 for each patient.

Patients were evaluated with the following tests: 12-lead scalar electrocardiogram, body weight, systolic and diastolic blood pressure, Doppler echocardiography, cardiopulmonary stress test, and carotid ultrasound. Heart rate was calculated from the electrocardiogram and systolic and diastolic blood pressure were measured by a cuff manometer after 15 min of supine rest.

Echocardiography

An ultrasound system equipped with a 2.5-MHz multifrequency transducer (Toshiba Aplio CV; Toshiba Corporation, Tokyo, Japan) was used for complete M-mode, two-dimensional, Doppler and tissue Doppler imaging (TDI) echocardiographic analyses. M-mode and two-dimensional recordings were made with the patients in the lateral recumbent position, according to the updated recommendations for chamber quantification (10). The investigator reading the echoes was blinded to the study protocol. The methods are described in detail elsewhere (11). Measures of left ventricular (LV) architecture and function were assessed according to standard formulae. The following parameters of diastolic function were measured as the mean of three to five consecutive beats: diastolic transmitral peak velocities, the maximal early diastolic flow velocity (E)/maximal late diastolic flow velocity (A) ratio, the isovolumic relaxation time, and mitral deceleration time. Quantitative diastolic data were derived from TDI analysis. The sample volume (4 mm^3) was placed in the LV basal portions of the anterior, inferior, septal, and lateral walls (using the two and four chambers images) (12). The following parameters were derived early (E′) and late (A′) diastolic velocities and the E′/A′ ratio. Peak early diastolic mitral annular velocity provides a relatively load-insensitive measure of LV relaxation and progressively decreases with increasing severity of diastolic dysfunction.

Cardiopulmonary exercise test

Symptom-limited cardiopulmonary exercise testing (Treadmills Rammill Series; Morgan Italia, Bologna, Italy) was performed, according to the Cornell-modified multistage treadmill protocol (2 min step increments) with a commercially available equipment (Benchmark exercise test system, Morgan Italia) (13). Measurements of oxygen consumption (VO_2), were taken at rest and during exercise using a moving average of eight breaths. During each stage of exercise, data on heart rate and rhythm and blood pressure were collected. All patients were encouraged to exercise until they felt unable to continue because of dyspnea and/or fatigue. The maximum VO_2 was defined as the highest VO_2 value measured (peak VO_2). The anaerobic threshold was determined by the V-slope method, in which CO_2 production is plotted as a function of VO_2, and the break point at which CO_2 production increases more rapidly than VO_2 is taken as the anaerobic threshold (13).

Carotid ultrasound examination was performed in each subject using a validated protocol (14), with a 7.5-MHz multifrequency linear-array probe (Toshiba Aplio CV; Toshiba Corporation, Tokyo, Japan). Briefly, ultrasound examination was made with the subject in a supine position, with a slight rotation of the neck. Both the common and the internal carotids were scanned bilaterally by experienced vascular sonographers (U.O., L. Sal.), blinded to the subject's clinical features. The probe was placed along the vessel axis, and carotid arteries were explored with longitudinal (anterior, lateral, and posterior) and transverse scan. The probe was manipulated so that the near and far walls were parallel to the probe, and the lumen diameter was maximized in the longitudinal plane. The origin of carotid bifurcation was identified and served as a reference point for the start of the measurements. Intima-media thickness (IMT) values were obtained at the far wall on both sides of the carotid bifurcation using a digital caliper and a semiautomated edge detection system (Toshiba), which provided the average thickness across a 1-cm segment. The ultrasonographic pictures were also stored according to DICOM standards for subsequent supervision of adherence to the imaging protocol by a senior investigator (U.O.) with more than 10 yr of experience in ultrasound examination of carotid vessels.

Statistics

Data were handled, stored, and analyzed using the SPSS package (SPSS Inc., Chicago, IL). Independent-sample *t* test for unpaired data, linear regression analyses, and Fisher's exact test (two tailed) were used when appropriate. Multiple regression analysis with the backward elimination procedure was carried out. To evaluate determinants of altered diastolic filling, exercise performance, and IMT, the following clinical and hormonal characteristics of the patient groups were the independent variables tested: mTSH, number of episodes of TSH greater than 4.0 or less than 0.5 mU/liter and their summation, the average of all TSH values

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TABLE 1. Clinical and laboratory characteristics of the patients with CH and controls

	Controls	CH	P
No. of patients	32	32	ns
Sex (male/female)	11/21	11/21	ns
Chronological age (yr)	19.0 ± 0.5	18.1 ± 0.2	ns
BMI (kg/m^2)	21.3 ± 0.5	22.9 ± 0.7	ns
Body surface area (m^2)	1.6 ± 0.04	1.5 ± 0.03	ns
Heart rate (beats/min)	77 ± 1.9	74 ± 1.8	ns
Systolic blood pressure (mm Hg)	112 ± 0.6	113 ± 1.8	ns
Diastolic blood pressure (mm Hg)	76 ± 1.5	74 ± 1.3	ns
Total cholesterol (mg/dl)	151.8 ± 0.7	148.8 ± 5.1	ns
Triglycerides (mg/dl)	64.9 ± 0.8	62.6 ± 5.3	ns
TSH (mU/liter)	1.8 ± 0.3	2.95 ± 0.7	ns
Free T_3 (pg/ml)	3.2 ± 0.2	2.9 ± 0.1	ns
FT4 (pg/ml)	11.4 ± 0.5	12.3 ± 0.3	ns
L-T4 (μg/kg·d)		2.1 ± 0.06	
Pubertal mean TSH (mU/liter)[a]		3.8 ± 0.3	
Pubertal mean FT4 (pg/ml)[a]		15.3 ± 0.3	
Pubertal mean L-T4 (μg/kg·d)[a]		2.5 ± 0.2	

Data are expressed as mean ± SEM. ns, Not significant.

[a] Mean values from the onset of puberty to the time of the study.

greater than 4.0 mU/liter and less than 0.5 for each patient, free T_3, FT4, heart rate, and systolic and diastolic blood pressures. A univariate $P < 0.05$ was required for each independent variable to enter the equation. Data are reported as mean ± SEM.

Results

As expected, the study groups were matched for age, sex, and BMI. There were no differences in baseline blood pressure and heart rate (Table 1). LV architecture and systolic function were also similar in the two study groups, whereas patients with CH exhibited impaired LV diastolic function. In particular, there was evidence for a mild impaired relaxation pattern (15), documented by prolonged isovolumic relaxation and mitral deceleration time, and reduced E/A and E′/A′ ratio (Table 2). CH patients did not report any reduction of daily activities, compared with controls. However, exercise capacity and cardiopulmonary performance were significantly lower in patients with CH, compared with normal controls, as documented by reduced values of peak VO_2 consumption (−19% *vs.* controls), anaerobic threshold, and workload (Table 2). Both common and internal carotid IMT mean values were significantly increased in congenitally hypothyroid patients, compared with controls (Table 2, $P < 0.0001$ and 0.001 *vs.* controls, respectively). Follow-up TSH evaluations revealed frequent episodes of overtreatment and undertreatment. Specifically, an average of 17% of TSH evaluations detected subclinical hyperthyroidism (TSH < 0.5 mU/liter) and 38% detected subclinical hypothyroidism (TSH > 4.0 mU/liter) in our patient population.

The degree of TSH derangement among the patients was large and ranged between 0.01 and 70 mU/liter. However, whereas

TABLE 2. Echocardiographic and cardiopulmonary data and carotid IMT of patients with CH and controls

	Controls	CH	P
LV-IS diastole (mm)	8.5 ± 0.2	8.5 ± 0.2	ns
LV-PW diastole (mm)	8.3 ± 0.3	7.9 ± 0.2	ns
LV-ED dimension (mm)	47.6 ± 0.9	46.7 ± 0.7	ns
LV-ES dimension (mm)	28.0 ± 0.8	27.9 ± 0.7	ns
LV mass index (g/m^2)	128.0 ± 7.0	112.0 ± 7.0	ns
LV ejection fraction (%)	61.0 ± 4.0	60.0 ± 5.7	ns
LV end systolic stress (g/m^2)	54.0 ± 2.0	56.0 ± 3.0	ns
Isovolumic relaxation time (msec)	72.7 ± 1.9	78.7 ± 1.6	<0.05
Mitral deceleration time (msec)	253.0 ± 14.0	309.0 ± 5.0	<0.001
Mitral E/A	1.8 ± 0.08	1.59 ± 0.06	<0.05
Mitral E′/A′ TDI	1.93 ± 0.09	1.41 ± 0.1	<0.01
Peak VO_2 (ml/kg/min)	37.3 ± 1.9	30.3 ± 1.7	<0.01
Peak anaerobic threshold (ml/kg/min)	35.6 ± 2.2	28.7 ± 1.9	<0.01
Peak workload (W)	293 ± 22	225 ± 17	<0.01
Peak systolic blood pressure (mm Hg)	140 ± 5.8	142 ± 2.4	ns
Peak heart rate (beats/min)	159 ± 5.0	152 ± 4.5	ns
IMT, common carotid artery (mm)	0.62 ± 0.03	0.68 ± 0.02	<0.0001
IMT, internal carotid artery (mm)	0.61 ± 0.04	0.68 ± 0.02	<0.001

All values are expressed as the mean ± SEM. ns, Not significant; IS, interventricular septum; PW, posterior wall thickness; ED, end diastolic; ES, end systolic; E′/A′ TDI, E/A ratio derived from TDI.

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episodes of severe hypothyroidism, defined as a TSH value above 10 mU/liter, occurred in all patients and more frequently within the first 10 yr of age, TSH values less than 0.1 were very rare and were found in only six patients, most of whom were over the age of 10 yr. We also tried to identify a critical developmental period at which the heart is most susceptible to damage induced by thyroid dysfunction. To this aim, we subdivided the patient population into four groups according to the patients' age (<5, 5–10, 10–15, 15–18 yr). The mean TSH levels were higher during the first 10 yr of follow-up than during adolescence (after the age of 10 yr). Specifically, TSH was 6.6 ± 3.2 mU/liter, compared with 3.5 ± 1.9 mU/liter ($P = 0.0001$). In particular, the age between 1 and 5 yr was characterized by the highest mean TSH values (9.0 ± 5.3 mU/liter, $P < 0.0001$ *vs.* all other ages). Interestingly, the mean TSH between 1 and 5 yr of age was negatively correlated with VO_2 max at the time of the study ($r = -0.63$, $P = 0.0003$). Finally, the number of episodes of both hyper- and hypothyroidism across the age intervals was significantly lower after the age of 15 yr ($P < 0.003$ *vs.* other ages), suggesting that an intensive control is more advisable until the end of puberty

No significant differences in cardiovascular parameters were observed when the patients were subdivided on the basis of etiological defects, athyreosis, ectopic, and eutopic gland nor between patients with mild or severe CH at diagnosis (data not shown). Furthermore, we did not find any significant correlation between serum T_4 levels at the time of diagnosis and the long-term cardiac function

Univariate analysis showed a significant correlation between LV diastolic filling indexes and cardiopulmonary test variables (Fig. 1). Specifically, E′/A′ ratio was directly correlated with peak VO_2, isovolumic relaxation time with VO_2 at anaerobic threshold and peak workload, and mitral deceleration time with peak workload. Variables derived from carotid ultrasound did not correlate with biochemical indexes; the only correlation was found between IMT of common carotid artery and E′/A′ ratio.

Multiple regression analyses were performed in the patient population including all hormonal measures as independent variables. By these analyses, the number of episodes of subclinical hyper-and hypothyroidism, their summation, and mean TSH values during puberty were independent predictors of isovolumic relaxation time, mitral E′/A′, peak VO_2, peak anaerobic threshold, and peak workload (Table 3). The average of all TSH values greater than 4.0 and less than 0.5 mU/liter were not independent predictors of any of the cardiological parameters in the multivariate analysis. Taken together, our data suggest that cardiovascular abnormalities are not correlated with the degree of TSH deviation from the normal range but with the number of episodes of TSH derangement.

Discussion

The current study provides the first evidence that patients with CH present with abnormalities of the cardiovascular system including

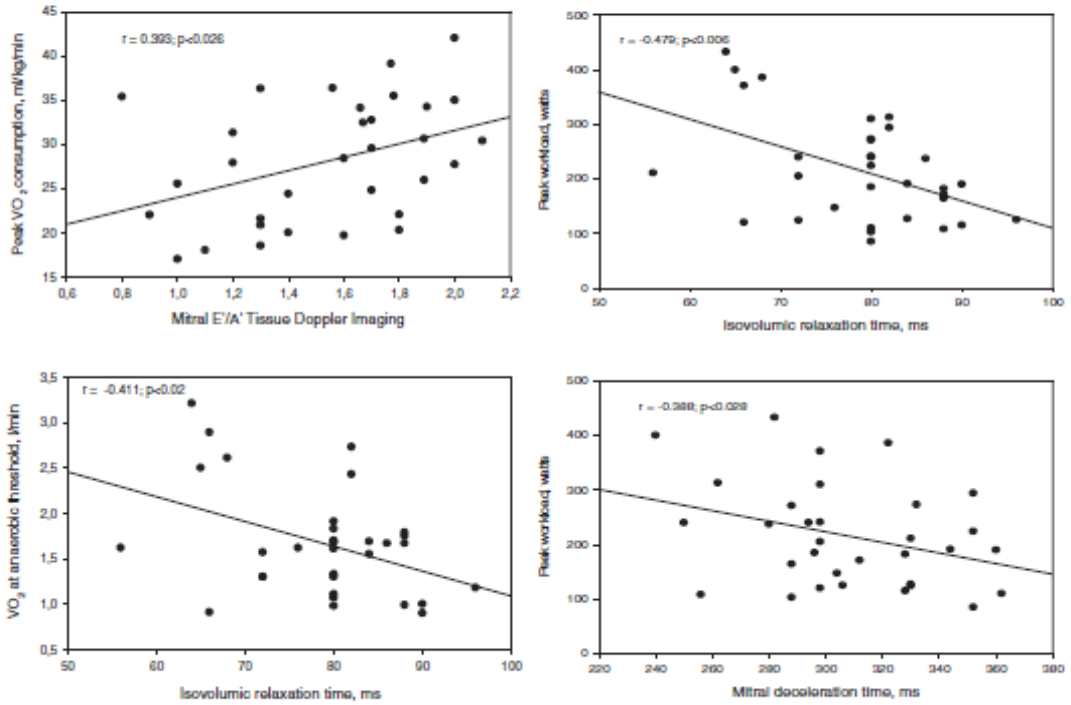

FIG. 1. Linear regression analyses between cardiopulmonary test parameters and indexes of LV diastolic filling.

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TABLE 3. Multivariate predictors of altered cardiovascular status

Dependent variable	Independent predictors	Multiple r
Isovolumic relaxation time	Mean TSH, number of episodes of hypothyroidism	0.61
Tissue Doppler mitral E′/A′ ratio	Number of episodes of hyper- and hypothyrodism, total episodes of inadequate treatment	0.75
Peak VO_2 consumption	Number of episodes of hyper- and hypothyrodism	0.65
Peak anaerobic threshold	Mean TSH, number of episodes of hypothyrodism	0.71
Peak workload	Number of episodes of hyper- and hypothyrodism, total episodes of inadequate treatment	0.69

an impairment of diastolic function, a reduction of exercise capacity and cardiopulmonary performance, and an increased IMT. Such abnormalities occur despite careful replacement therapy and appear related to unphysiological fluctuations of TSH levels, with attendant episodes of subclinical hyperthyroidism and, more frequently, subclinical hypothyroidism.

To our knowledge, long-term cardiac function has never been investigated in children and adolescents with congenital hypothyroidism detected by neonatal screening and treated from the first month of life. Few and conflicting data have been reported only in untreated neonates with congenital hypothyroidism. Specifically, cardiac function has been reported to be either normal (15, 16) or impaired (17, 18). Recent data suggest that at the time of diagnosis, neonates with CH may exhibit left and right ventricular systolic and diastolic dysfunction, which can be reversed by early L-T4 substitutive treatment (19).

The impairment of diastolic performance in CH is supported by a broad array of Doppler indexes and resembles the typical pattern of a mild relaxation abnormality (20). The relaxation phase is indeed an energy-dependent phase of LV filling, characterized by calcium reuptake toward the sarcoplasmic reticulum (21). LV relaxation is very sensitive to even mild energy depletion, and its impairment often precedes systolic dysfunction. Abnormal relaxation results in persistent pressure generation at end diastole and may thus lead to reduced LV distensibility, which in turn is known to contribute to exercise intolerance (22). Exercise intolerance is largely dependent on reduced stroke volume during exercise caused by the limited increase in the LV end-diastolic volume despite normal ejection fraction and the increased LV filling pressure and left atrial pressure during exercise. Such speculation is supported by the significant relationship between E′/A′ and peak VO_2. Heart rate response to exercise was not different between the two study groups, whereas aerobic work capacity and efficiency was equally impaired as indicated by lower VO_2 and anaerobic threshold. CH patients exhibited a decrease in not only exercise duration and maximal O_2 uptake but also anaerobic threshold. Exercise duration and maximal O_2 uptake are indexes of work capacity that may not be entirely objective because they may be limited by symptoms or patient motivations. The anaerobic threshold is the exercise level above which aerobic energy production is supplemented by anaerobic mechanism and is therefore more objective (23). Despite the fact that exercise capacity was impaired, CH patients did not experience any difference in daily activities, compared with healthy controls. This finding might be explained by the fact that most daily activities occur at low levels of oxygen consumption.

Congenitally hypothyroid patients also displayed a significant increase of carotid IMT, a surrogate marker of atherosclerotic disease (24). This alteration was present for each side on carotid axis in patients with CH and caused a diffuse and homogeneous increase of the arterial wall thickness in both the common and internal carotid arteries. In this regard, recent evidence support an independent association between thyroid function and carotid IMT. Specifically, in a large population-based survey, patients with low TSH levels (<0.3 mU/liter) had the highest IMT values (25). On the other hand, also subclinical hypothyroidism is associated with increased IMT in several cross-sectional analyses (26, 27). These observations provide a solid background for increased IMT in patients who present with frequent episodes of subclinical thyroid dysfunction.

Several lines of experimental evidence support the concept that subclinical thyroid dysfunction is associated with a broad array of cardiovascular abnormalities, which may confer enhanced risk for atherosclerosis, myocardial infarction, and cardiovascular death (4, 28–29). Specifically, we and others have previously demonstrated that subclinical hyperthyroidism is associated with increased heart rate, increased LV mass with mild concentric remodeling, reduced exercise performance, and impaired ventricular relaxation (30, 31). The cardiovascular phenotype of subclinical hypothyroidism is that of impaired diastolic function, altered systolic function under effort, and increased IMT (32, 33).

Taken together, the cardiovascular abnormalities displayed by patients with CH may well depend on the summation of multiple episodes of subclinical hypo- and hyperthyroidism. In fact, the cardiac phenotype of hypothyroid patients was characterized by impaired LV relaxation, reduced exercise capacity, and increased IMT. Such speculation is supported by the multivariate analysis that demonstrated that the number of episodes of overtreatment and undertreatment and their summation, and mTSH predicted the alterations of peak VO_2 consumption, peak VO_2 at anaerobic threshold, peak workload, and E′/A′ ratio. However, association does not necessarily imply a mechanistic link, and potential causes of cardiovascular abnormalities other than L-T4 treatment should be taken into account, such as fetal programming of adult cardiac function.

Our data are congruent with previous reports showing that congenitally hypothyroid patients often present with TSH serum concentration above or below the normality range, particularly during adolescence when treatment compliance becomes less regular, notwithstanding an accurate biochemical follow-up and frequent adjustments (7).

The prevalence of inadequate compliance, with increased levels of TSH, is particularly high in children and adolescents with CH. Indeed, inadequate control has been demonstrated in 74% of children older than 12 yr (7). To date, most reports have focused on the effects of over- or undertreatment on mental, psychomotor, and

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behavioral outcome in children with CH. Indeed, low IQ scores, increased level of both anxiety and inattention, and delayed progression during elementary school have been shown to correlate with the number of episodes of undertreatment (6, 25, 26). On the contrary, the effects of moderate overtreatment are still debatable, with both lower verbal scores or no impairment of cognitive development being reported (7, 27).

The reasons underlying episodes of overtreatment may depend on the current recommendations for treatment of CH, that increased L-T4 dose from 5–8 to 10–15 μg/kg·d to achieve a more rapid normalization of T_4. Furthermore, the optimal therapeutic target aims at normal TSH levels with T_3 and T_4 levels in the upper normal range, thus potentially leading in the long term to phases of subclinical hyperthyroidism.

In view of a long-term replacement therapy with L-T4, one must be aware that frequent episodes of subclinical hypothyroidism and hyperthyroidism may occur and should be avoided to prevent cardiovascular abnormalities. Moreover, a careful cardiovascular follow-up should be performed, and future studies will clarify whether these abnormalities may result in clinically relevant cardiovascular diseases.

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5.3 Effects on long term L-Thyroxine treatment on endothelial function and arterial distensibility in young adults with congenital hypothyroidism

EFFECTS OF LONG-TERM L-THYROXINE TREATMENT ON ENDOTHELIAL FUNCTION AND ARTERIAL DISTENSIBILITY IN YOUNG ADULTS WITH CONGENITAL HYPOTHYROIDISM

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EFFECTS OF LONG-TERM L-THYROXINE TREATMENT ON ENDOTHELIAL FUNCTION AND ARTERIAL DISTENSIBILITY IN YOUNG ADULTS WITH CONGENITAL HYPOTHYROIDISM

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Abstract

Objective: Patients with Congenital Hypothyroidism (CH) display subclinical abnormalities of cardiovascular system that are related to unphysiological fluctuations of TSH levels and occur despite careful replacement therapy.

Design: The aim of the present case-control study was to evaluate the effects of long-term levothyroxine (L-T4) replacement therapy on the vascular district in CH patients by assessing endothelial function with flow mediated dilation (FMD) and brachial arterial distensibility (BAD) with the measurement of the coefficient of distensibility (DC).

Methods: Thirty-two young adults with CH aged 18.9±0.2 years and thirty-two age and sex-matched controls performed brachial Doppler ultrasound examination to measure FMD and DC at the time of the study. Hypothyroidism was diagnosed by neonatal screening and L-T4 treatment was initiated within the first month of life.

Results: Compared to healthy controls CH patients displayed a significantly reduced brachial artery reactivity with lower FMD values (8.9±5.7 vs 14.1±5.1% p=0.003) and decreased vascular distensibility (24.6±1.6 vs 27.3±3 $kPa^{-1}x10^{-3}$, p<0.0002). Linear regression analysis revealed that both total and pubertal mean TSH and number of episodes of under-treatment resulted independent determinants of FMD and DC. Pubertal mean TSH resulted the best predictor of both FMD and DC (r=0.81 and r=0.87 respectively, p<0.001).

Conclusions: Young adults with CH treated with long-term L-T4 replacement therapy may have a significant impairment of both FMD and DC. Our data suggest that high TSH levels, inadequately corrected by L-T4 replacement therapy in CH patients in particular during pubertal period, can exert important effects on the elastic and functional vessel properties.

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Introduction

Thyroid hormone has important effects on cardiovascular system (1). Overt hypothyroidism represents a well known risk factor for atherosclerotic disease. Specifically, it is characterized by endothelial dysfunction, increased peripheral vascular resistance, early development of structural atherosclerotic lesions and appearance of clinical vascular events (1-3). Moreover, cardiovascular abnormalities, such as left ventricular diastolic impairment, increase of carotid intima-media thickness (IMT) and endothelial dysfunction, have been also reported in patients with subclinical hypothyroidism (SH) (4-7). In this regard, two recent meta-analyses found that SH is associated with an increased risk of coronary heart disease (8, 9), while therapeutic trials with L-thyroxine (L-T4) treatment in patients with SH showed a beneficial effect on both functional and structural atherosclerotic markers (10-14). All the determinants of the cardiovascular risk in patients with thyroid function abnormalities are not fully understood, even if hypercholesterolemia and a direct effect of thyroid hormones on arterial wall are certainly involved (1).

Congenital hypothyroidism (CH) represents one of the most common endocrine diseases in newborns, with a worldwide incidence of 1:3000 to 1:4000 (15, 16). We recently provided the first evidence that patients with CH display early cardiac involvement as showed by an impairment of diastolic function and a reduction of exercise capacity and cardiopulmonary performance (17). Such abnormalities occurred despite careful replacement therapy and resulted associated with fluctuations of TSH levels and episodes of subclinical hypothyroidism. Episodes of subclinical hyperthyroidism were less frequently involved.

Endothelial dysfunction, is an early event in the atherogenesis and can precede the appearance of structural vascular changes as the increase of carotid intima-media thickness. It can be usefully studied by flow mediated dilation (FMD) and can be used to predict clinical outcome in high-risk cohorts of patients (18).

The vascular function, moreover, can be also evaluated in an early stage of the atherogenesis by the measurement of brachial artery distensibility (BAD), which represents a specific index of the vessel elastic properties and is inversely related to arterial stiffness.

The aim of the present study was to assess endothelial function and brachial artery distensibililty in young adults with CH who were followed longitudinally from the first weeks of life, in comparison to

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matched euthyroid subjects and evaluate whether unphysiological fluctuation of TSH, associated with a long-term L-T4 replacement therapy, might place them at an increased risk of early atherosclerotic changes.

Subjects and Methods

Patient population

Thirty-two young adults (21 females, 11 males) affected with CH, aged 18.9±0.2 years, participated in the study. All patients, were diagnosed by neonatal screening and were followed longitudinally, from the time of diagnosis of CH to the time of the study. The diagnosis was confirmed by serum thyroid function tests. L-T4 replacement therapy was started immediately after the first evaluation, at a mean age of 26±0.9 days (range 12-30 days) and at a mean initial dose of 6.5±0.1 µg/kg/day. Replacement therapy was modified during follow-up according to clinical and hormonal evaluation in order to maintain serum TSH in the normal range and serum FT4 in the upper normal range. The etiological diagnosis of CH was made on the basis of 99m Tc-pertechnetate or iodine-123 thyroid scans at the time of diagnosis or at the age of 3 years, after the withdrawal of L-T4 therapy for six weeks. Cases were classified into three groups: athyreosis (n=7), ectopic (n=18) and eutopic gland (n=7). At study entry, all subjects had completed their pubertal development and females had regular menstrual cycles. All patients had already reached their adult height (defined as a growth of less than 1.0 cm/yr during the preceding year) (19). Serum TSH and thyroid hormones, and routine blood analysis were periodically assessed (every six months from the diagnosis of hypothiroidsm). Three patients were mild smokers (less than 10 cigarettes per day) and three were moderate drinkers (beer or wine occasionally). Previous or current cardiovascular, respiratory, renal or other chronic diseases as well as obesity were considered exclusion criteria.

Thirty-two healthy young adults comparable for age, sex, body mass index and physical activity participated in the study as controls.

Informed consent was obtained from all patients or from their parents for the patients younger than 18 years. The study was approved by the Ethical Committee of the Federico II University.

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Study protocol

At study entry, all subjects underwent height, weight, BMI, heart rate, systolic (SBP) and diastolic blood pressure (DBP) measurements. TSH, thyroid hormones, total cholesterol and triglycerides were also evaluated.

Vascular function was measured in each patient at the time of the study by the measurements of FMD and DC.

In CH patients mean TSH and FT4 values were calculated from all the samples carried out during the study from the age of 1 year (mean total TSH and mean total FT4) to the time of the study. Pubertal mean TSH was calculated on the samples collected from the age of the first sign of pubertal development (breast stage 2 for females and testicular volume of 4 ml for males) to the time of the study . Mean L-T4 dose taken in the same periods was also calculated.

Based on the normal TSH range in our laboratory (0.5-4.0 mU/L) we also calculated an index of over-treatment (number of episodes with plasma TSH <0.5 mU/L) and an index of under-treatment (number of episodes of plasma TSH >4.5 mU/L).

Measurements below 1 year of age were excluded since TSH plasma levels were still above the normal range in a vast majority of patients.

Flow mediated dilation

Brachial artery reactivity was evaluated in each subject using validated protocol (20), with a 7.5 MHz multifrequency linear-array probe (Aplio XG imaging system, Toshiba, Japan). All subjects were evaluated in a quiet, temperature-controlled room and, from the day before the examination, have withdrawn cigarette smoking. Measurements began approximately at 12.30 pm. After resting for 10 min in a supine position, electrocardiographic leads were connected and a sphygmomanometer cuff was placed on the right arm. The brachial artery was imaged approximately 2-5 cm proximal to the antecubital crease in a longitudinal axis, and the brachial artery diameter, from the intima-lumen interface on the near wall to the media-adventitia interface on the far wall, was measured at end-diastole cycle, on the electrocardiographic R-wave. Endothelium-dependent vasodilatation was assessed by measuring the maximum increase in brachial artery diameter during reactive hyperemia created by the inflation of the cuff (250 mmHg for 5 min) placed on the

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right arm. After sudden cuff deflation, flow velocity indexes were measured in the first 15 s, then brachial artery diameter was measured at least four times during the next 90 s. FMD resulted from the formula: FMD = [(post-hyperaemia diameter – baseline diameter)/ baseline diameter] x 100. After subject had rested for at least 10 min, nitroglycerin spray (0.6 mg) was sublingually administered in order to assess endothelium-independent vasodilatation (NMD). Peak nitroglycerin vasodilatation occurs about 3 min after nitroglycerin administration. Both flow velocity measurements and brachial artery diameter were recorded four times during this period. NMD resulted from the formula: NMD = [(postnitroglycerin diameter – baseline diameter)/ baseline diameter] x 100.

Brachial artery distensibility

Coefficient of distensibility of brachial artery (DC) was assessed for the evaluation of arterial stiffness. All measurements were done after an 8-hour fasting. All the subjects rested in a supine position for 15 minutes in a quiet, temperature-controlled room before the measurements. DC was obtained by a single investigator using the same ultrasound system (AplioToshiba) equipped with a 7.5-MHz linear array probe under electrocardiographic monitoring. The mean of 3 consecutive measurements was used in the analyses as recommended. The brachial artery was imaged approximately 2-5 cm proximal to the antecubital crease, in a longitudinal axis, and vessel diameters were measured in M-mode: the lowest end-diastolic arterial diameter (Dd) on the electrocardiographic R-wave, the highest end-systolic arterial diameter (Ds) on the electrocardiographic T-wave and the diameter change during cardiac cycle (ΔD, defined as Ds-Dd). Brachial artery pulse pressure (ΔP), defined as systolic minus diastolic blood pressure, was measured by a sphygmomanometer and expressed in kPa. Finally, DC was calculated as $(2\Delta D/Dd)/\Delta P$ ($kPa^{-1} \times 10^{-3}$) (21).

Statistical analysis

Data are reported as mean±D.S., unless otherwise specified. The statistical analysis was performed using the U-Mann-Whitney rank-sum test. P value less than 0.05 was considered statistically significant.

Linear regression analysis was performed using FMD and DC as dependent variables and the following as independent variables: total and pubertal mean TSH, number of episodes of TSH >4.5 or <0.5 mU/l.

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Results

At study entry, no differences were detected in clinical and laboratory findings as BMI, heart rate, blood pressure, total cholesterol and triglycerides levels, thyroid hormones between CH patients and controls. (Table 1)

Compared to the control group CH patients showed a significantly reduction in both mean FMD value 8.9±5.7 vs 14.1±5.1%, p=0.0003) and mean DC value (24.6±1.6 vs 27.3±3 $kPa^{-1}x10^{-3}$, p<0.0002) (Fig. 1). In contrast, no differences were detected in NMD values between CH patients and controls (20.2±2.3 vs 22±3.5%).

Linear regression analysis revealed that independent determinant of FMD and DC were mean total TSH (r= 0.67 and r=0.79 respectively, p<0.001), mean pubertal TSH (r=0.81 and r=0.87 respectively, p<0.0001) (Fig. 2, 3), total number of episodes of hypothyroidism (r=0.58 and r=0.62, p<0.02 and p<0.01 respectively) and pubertal episodes of hypothyroidism (r=0.63 and r=0.67, p<0.002 and p<0.001 respectively) (Table 2). Both FMD and DC resulted more significantly associated with pubertal TSH (r=0.81 and r=0.87, respectively) in respect to the other independent predictors obtained (mean total TSH and number of episodes of hypothyroidism).

No significant correlation was detected between both FMD and DC and the number of episodes of subclinical hyperthyroidism in CH patients. Moreover, no significant differences in vascular function and structure were observed dividing the patients on the basis of detected aetiological defects or on the basis of the severity of CH at diagnosis (data not shown).

Discussion

The results of the current case-control study indicate that young adults with CH treated with long-term L-T4 replacement therapy, present early vascular alterations, as demonstrated by the presence of endothelial dysfunction and arterial distensibility impairment. In fact, as compared to healthy controls, CH patients displayed a significant reduction of both FMD and DC values.

The impairment of functional and elastic vessel properties showed a strong correlation with higher mean values of TSH during the overall follow-up and in particular during the pubertal period. Moreover, the total number of subclinical hypothyroidism episodes due to inadequate LT4 replacement therapy during the

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study and the total number during the pubertal period represent a predictive factor for the reduction of FMD and the impairment of the arterial distensiblity.

Of course, episodes of inadequate LT4 replacement therapy may occur during long-term treatment of CH patients. Indeed, our CH patients experienced periods of subclinical hypothyroidism, particularly during adolescence, when the compliance to the treatment becomes less regular, not withstanding an accurate biochemical follow-up and frequent adjustments. These episodes were strongly correlated with the impairment of both FMD and DC observed at the time of the study. On the contrary, no relationship was observed between episodes of subclinical hyperthyroidism, less frequently detected in CH patients, and vascular abnormalities at the time of the evaluation.

These results are in agreement with our previous study documenting an impairment of diastolic function and cardiopulmonary performance in young CH adults associated with episodes of subclinical hypothyroidism (17).

Other studies have shown a strong positive relationship between serum TSH values and endothelial dysfunction in adults with subclinical hypothyroidism (10). In some cases, however, the impairment of endothelial function was not explained by the presence of the usual cardiovascular risk factors (22), thus suggesting that TSH is itself endowed with atherogenic activity or it may regulate vascular homeostasis.

In agreement to this hypothesis the presence of functional TSH receptor has been demonstrated in cardiomyocites (23), in human coronary artery smooth muscle cells (24) and in human endothelial cell (25). Moreover, recombinant human TSH administration has been shown to acutely impair endothelium-dependent vasodilatation (26). Nevertheless, the intimate mechanisms of the interaction between TSH and vascular system have yet to be completely clarified.

In conclusion, our data indicate that young adults with CH treated with long-term L-T4 replacement therapy may have repeated episodes of TSH increase that can modify vascular reactivity and arterial distensibility by mechanisms not yet completely explained.

However, endothelial dysfunction and brachial artery distensibility are potentially reversible events, thus long-term studies are needed to clarify if these vascular abnormalities can be reversed after a sustained normalization of TSH concentration. In the meantime we suggest careful follow-up with frequent dosage adjustment to avoid episodes of undertreatment, particularly frequent during adolescence, in order to prevent

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early atherosclerotic abnormalities. Moreover, the usefulness of systematic noninvasive cardiovascular screening in this population should be considered.

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Legend to the figures

Figure 1 Flow mediated dilation (FMD) (A) and coefficient of distensibility of brachial artery (DC) (B) in CH patients and controls. The boxes show medians, 25th and 75th percentiles, and the whiskers represent the highest and lowest values

Figure 2 Linear regression analysis between flow mediated dilation (FMD) and total mean TSH (r = 0.67 B = -1.09) (right) and pubertal mean TSH (r = 0.81 B = -1.25) (left)

Figure 3 Linear regression analysis between coefficient of distensibility of brachial artery (DC) and total mean TSH r = 0.79 B = -0.63 (right) and pubertal mean TSH r = 0.87 B = -0.73 (left)

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Table 1 Clinical and laboratory characteristics of CH patients and controls subjects at study entry.

	CH	Controls	p
Nr of patients	32	32	ns
Sex (M/F)	11/21	11/21	ns
Age (yr)	18.9±0.2	19.5±0.5	ns
Body Mass Index (kg/m^2)	21.4±0.5	21.9±0.7	ns
Heart rate (beats/min)	88±3	84±3	ns
Systolic blood pressure (mmHg)	113±2	110±2	ns
Diastolic blood pressure (mmHg)	73±1	75±2	ns
Total cholesterol (mg/dl)	149±5	150±6	ns
Triglycerides (mg/dl)	62±6	57±5	ns
L-T4 (μg/kg/day)	2.1±0.07	-	-
TSH (mIU/L)	2.7±0.3	1.8±0.3	ns
FT4 (pg/ml)	12.3±0.3	11.4±0.5	ns

Data are expressed as mean±S.E.M; ns: not significant.

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Table 2 Univariate predictors of impaired flow mediated dilation (FMD) and coefficient of distensibility of brachial artery (DC).

Dependent variables	Independent predictors	r	p
FMD	Mean total TSH	0.67	<0.001
	Pubertal TSH	0.81	<0.0001
	Number of episodes of hypothyroidism	0.58	<0.02
	Number of pubertal episodes of hypothyroidism	0.63	<0.002
DC	Mean total TSH	0.79	<0.001
	Pubertal TSH	0.87	<0.0001
	Number of episodes of hypothyroidism	0.62	<0.01
	Number of pubertal episodes of hypothyroidism	0.67	<0.001

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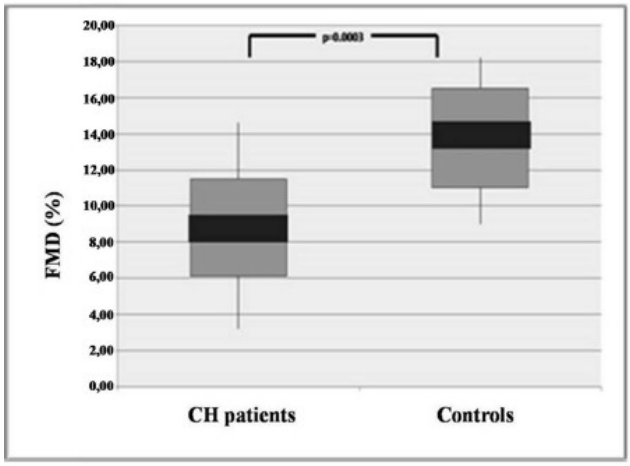
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20,00
18,00
16,00
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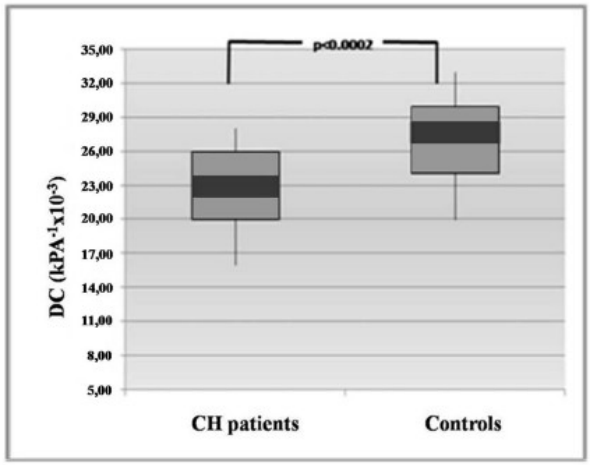
12,00
10,00
8,00
6,00
4,00
2,00
0,00
FMD (%)
CH patients
Controls


p<0.0002
35,00
32,00
29,00
26,00
23,00
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17,00
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8,00
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DC (kPA-1x10-3)
CH patients
Controls

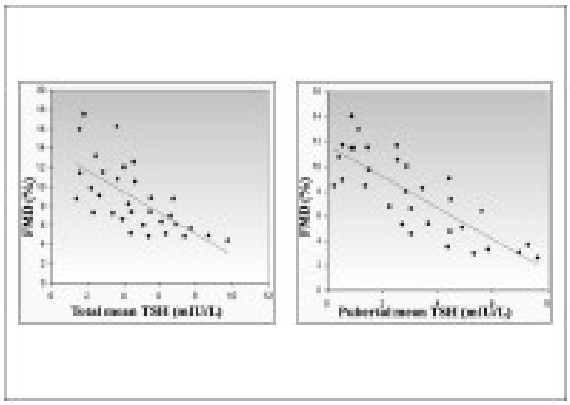

Figure 2
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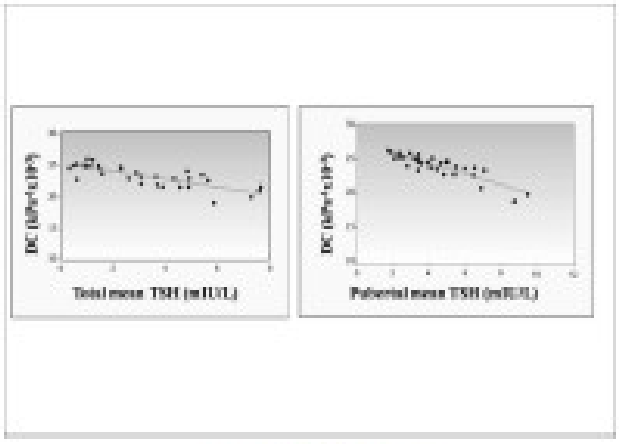

Figure 3
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5.4 Prospective evaluation of the natural course of idiopathic subclinical hypothyroidism in childhood and adolescence

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CLINICAL STUDY

Prospective evaluation of the natural course of idiopathic subclinical hypothyroidism in childhood and adolescence

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Abstract

Objective: To prospectively evaluate the course of subclinical hypothyroidism (SH) in children and adolescents with no underlying diseases and no risk factors, which might interfere with the progression of SH.

Design: Clinical status, thyroid function, and autoimmunity were prospectively evaluated at entry and after 6, 12, and 24 months in 92 young patients (mean age 8.1 ± 3.0 years) with idiopathic SH.

Results: During the study, mean TSH levels showed a trend toward a progressive decrease while FT_4 levels remained unchanged. Overall, 38 patients normalized their TSH (group A): 16 patients between 6 and 12 months, and 22 patients between 12 and 24 months. Among the remaining 54 patients, the majority maintained TSH within the baseline values (group B), whereas 11 exhibited a further increase in TSH above 10 mU/l (group C). Baseline TSH and FT_4 levels were similar in the patients who normalized TSH, compared with those with persistent hyperthyrotropinemia. Even in the patients of group C, both TSH and FT_4 at entry were not different with respect to those of groups A and B. No patients showed any symptoms of hypothyroidism during follow-up and no changes in both height and body mass index were observed throughout the observation period.

Conclusions: (a) The natural course of TSH values in a pediatric population with idiopathic SH is characterized by a progressive decrease over time; (b) the majority of patients (88%) normalized or maintained unchanged their TSH; and (c) TSH changes were not associated with either FT_4 values or clinical status or auxological parameters.

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Introduction

Subclinical hypothyroidism (SH) is a condition of moderate thyroid failure characterized by normal circulating levels of thyroid hormones with mildly elevated TSH serum concentrations. SH is a common clinical problem in adulthood and elderly, whereas its prevalence is distinctly lower in children and adolescents (1).

The natural course of SH in aged patients has been reported to be characterized by frequent normalization of TSH elevation, whereas the risk of progression to overt hypothyroidism was significantly greater in the ones with high TSH concentrations at baseline (2). Data concerning the natural evolution of SH in childhood and adolescence are very scanty. According to one of the few available follow-up studies on juvenile SH, this may be a benign and remitting process with a very low risk of evolution toward frank hypothyroidism (3).

All the available reports on the spontaneous evolution of SH in both aged and young patients have been based, up to now, on unselected study populations including patients with either thyroid disorders or other pathological causes that are well known to be able to affect SH development and evolution (1, 2, 4–7).

In the present multicenter study, we have prospectively investigated during a 2-year follow-up the spontaneous changes in TSH and FT_4 values in a pediatric population consisting of only patients with 'idiopathic' SH, i.e., without non-thyroidal diseases and with no previous thyroid disorders or therapies affecting thyroid gland. The aim of our study design was to prospectively evaluate for the first time the natural course of SH in children and adolescents with no underlying diseases and no risk factors that might interfere with the progression of SH.

Patients and methods

Study population and design

Ninety-two selected patients (50 boys) with 'idiopathic' SH were enrolled according to well-assessed inclusion and exclusion criteria.

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All of them were younger than 15 years (mean age 8.1±3.0 years, range 5.0–14.9) and had been referred to our pediatric endocrine clinics from the community by their pediatricians because of the incidental finding of elevated TSH concentrations in their usual annual checkup that included also TSH measurement. Out of them, 67 were prepubertal (72.8%) and 25 were pubertal (27.2%). All of them were of good clinical status and were not affected by any non-thyroidal illnesses. No patients exhibited either palpable goiter or symptoms generally associated with thyroid hypofunction. Thyroid ultrasonography (US) was normal and thyroid autoimmunity was negative in all the cases at the time of admission. None of them was taking iodinated drugs, lithium salts, glucocorticoids, or antiepileptic agents. Clinical history of all the patients did not evidence any antecedents of either neck radiation therapy or false positivity at congenital hypothyroidism screening. The study was performed in non-iodine-deficient areas. Therefore, in the entire study population, all the etiological causes of SH had been excluded at the time of admission.

All these patients with 'idiopathic' SH were studied as outpatients. SH was defined by elevated TSH concentrations (5–10 mU/l) in the presence of normal FT_4 concentrations (10.3–24.4 pm/l). Two measurements of these hormones with an interval of 1–3 months were required to enter in the follow-up period. Patients were prospectively evaluated 6, 12, and 24 months after their admission, and at each visit clinical and hormonal (TSH and FT_4) data and thyroid autoimmunity status were assessed. Thyroid US was reevaluated at the end of the 24-month follow-up in all the patients. In the patients who exhibited during follow-up a further increase in TSH levels above 10 mU/l, at the end of follow-up, L-T_4 treatment was begun, according to our guidelines (8) and other recommendations (9).

Methods

Thyroid function tests were determined in the fasting status and performed in the same laboratory for each subject. TSH and FT_4 were determined by high specific fluorometric immunoassays. Intra- and interassay variations were less than 10%.

Thyroid autoimmunity was studied by the measurement of serum levels of thyroid peroxidase antibodies (TPOAb) and the titer was considered positive for values higher than 20 U/ml.

Thyroid US was performed in all subjects with a high-resolution 7.5 MHz linear transducer. The analysis of thyroid volume and echogenicity in each patient was assessed by the same operator both at entry and at the end of follow-up.

Clinical evaluation aimed to ascertain the existence of symptoms and/or signs of hypothyroidism.

Auxological assessment was based on height (*H*) measurement and body mass index (BMI) calculation.

Standing *H* was measured with a Harpenden stadiometer (Holtain Ltd, Crymych, Dyfed, UK). BMI was calculated as weight divided by height squared (kg/m^2). To allow the comparison between different ages and genders, *H* and BMI were expressed as S.D. scores (SDS) according to the standards assessed by Cacciari *et al.* (10).

Statistical analysis

Results are expressed as mean values; variability is indicated by S.D. and/or value range. For comparisons of two means, Student's *t*-test (normally distributed data) and the Mann–Whitney *U*-test (non-parametric data) were used. Frequency rates were compared by χ^2 test. Correlations between quantitative variables were assessed using Pearson's correlation analysis. The level of significance was set at 0.05.

This study design was approved by the ethical committees of our hospitals, and patients and/or their parents gave their informed consent. Appropriate consents for this study were also obtained from the Study Group for Thyroid Diseases of the Italian Society for Pediatric Endocrinology and Diabetology.

Results

Changes in thyroid tests during follow-up

Average initial TSH concentrations significantly lowered during the first 6 months of follow-up and still further during the subsequent months (Table 1). The mean decrement in TSH concentrations from baseline to the end of follow-up was 1.0±2.1 mU/l. TSH decrement in the overall series was unrelated to either sex or age or pubertal status or concomitant FT_4 changes.

Mean FT_4 levels did not significantly change throughout the follow-up period (Table 1). In all the patients, FT_4 levels remained within normal range at each time.

Thyroid US at the end of the observation period revealed significant changes compatible with Hashimoto's thyroiditis in only two patients, while in the remaining ones no significant alterations were found. TPOAb remained undetectable during the entire follow-up in all patients but two, i.e., the same two with US changes.

TSH normalization

Overall, 38 out of 92 patients normalized their TSH concentrations (<5 mU/l) during the 2-year observation period (group A): no patients during the first 6 months, 16 patients between 6 and 12 months, and 22 patients between 12 and 24 months (Table 2).

The fall in TSH values was similar in early and late normalizers.

Only two patients (5.3%) reverted to TSH values lower than 2 mU/l, while most normalizers (68.4%) achieved TSH levels between 3 and 4.3 mU/l. Among

Nevertheless, it is to be underlined that in our series only a minority of patients (5.3%) reverted to TSH values lower than 2 mU/l, whereas most normalizers (68.4%) achieved TSH levels between 3 and 4.3 mU/l, a result very similar to the one recently reported by Diez *et al.* (12). The short duration of follow-up in the present study, however, does not allow us to predict the long-term evolution of these patients. In the early normalizers of the present study, TSH normalization was confirmed 12 months later, i.e., at the end of follow-up period. However, to further characterize the spontaneous evolution of SH in children, more prolonged follow-up studies are necessary.

Finally, in our series both early and late normalizers exhibited an early decrease in TSH, whereas no significant decrease from baseline to 12 months was recorded in the patients who did not normalize at all their TSH. Early or late occurrence of TSH normalization in our study population was mainly conditioned by baseline TSH and FT_4 levels.

Among the possible factors influencing the inter-individual variations in serum TSH, mutations and polymorphisms which occur in the genes encoding the proteins involved in the TSH pathway could play a significant role. In this regard, the direct involvement of TSH receptor (*TSHR*) gene in the serum TSH concentration has been proven in some cases of SH, in which loss of function mutation has been identified (15, 16). However, a low prevalence of TSHR mutations has been recently reported in children with SH (17). In the present series, we did not examine *TSHR* gene because this was not the aim of our study.

In conclusion, (a) the natural course of TSH values in a pediatric population with idiopathic SH is characterized by a progressive decrease over time; (b) the majority of patients (88%) normalized or maintained unchanged their TSH; (c) TSH changes were not associated with any changes in either FT_4 values or clinical status or auxological parameters; and (d) TSH determination has no reason to be part of the routine checkup in children, apart from specific protocols.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Table 1 Changes in average (±S.D.) TSH and FT_4 concentrations at various times during the entire follow-up in the overall study population.

	Baseline	6 months	12 months	24 months
TSH (mU/l)	6.1±1.3	5.4±1.8*	5.3±2.4*	4.7±1.4*
FT_4 (pm/l)	14.3±3.4	14.2±3.1	14.0±3.5	14.0±2.5

*$P<0.01$ versus baseline TSH.

the remaining 54 patients who did not normalize TSH concentrations during the observation period (58.7%), the majority maintained their TSH within the baseline limits (5–10 mU/l) during the entire follow-up (group B), whereas 11 exhibited a further increase in TSH levels above 10 mU/l (between 10.5 and 15.0) despite persistently normal FT_4 levels and underwent L-T_4 therapy after the end of follow-up according to our study design (group C). In 2 out of these 11 patients, TSH increase was accompanied by both TPOAb detection and thyroid US features of Hashimoto's thyroiditis at the end of follow-up.

The percentages of patients with either decreasing or increasing or stable TSH values are scheduled in Table 2.

Factors influencing TSH normalization

Average baseline TSH concentrations (6.0±1.5 vs 6.2±1.1 mU/l) were very similar in the patients who normalized their TSH during follow-up compared with those with persistent hyperthyrotropinemia. Even in the patients of group C, both TSH and FT_4 at study entry (6.0±1.2 mU/l and 15.6±3.8 pm/l respectively) were not significantly different with respect to those of groups B (6.2±1.1 and 14.8±3.4 respectively) and A (6.0±1.5 and 13.6±3.4 respectively).

Patients who normalized their TSH late (24 months) showed higher initial TSH levels (6.2±1.7 vs 5.8±1.3 mU/l, $P<0.0125$) and lower initial FT_4 levels (12.9±3.3 vs 14.7±3.3 pm/l, $P<0.05$) with respect to those who showed early normalization (12 months). However, even the patients who were late to normalize their TSH exhibited a significant decrease in TSH values from baseline to 12 months (from 6.2±1.7 to 5.0±1.6 mU/l, $P<0.05$), whereas no significant decrease from baseline to 12 months was observed in the patients who did not normalize their TSH during the entire follow-up.

Table 2 Percentages of patients who normalized their TSH (<5 mU/l) at various times during follow-up (group A) of the ones who maintained their TSH within the initial 5–10 mU/l limits (group B) and of those who exhibited an increase in TSH values above 10 mU/l (group C).

Groups (%)	6 months	12 months	24 months	Total
A	0	17.4	23.9	41.3
B	100	83.6	46.7	46.7
C	0	0	12.0	12.0

The prevalence of patients who normalized their TSH during the follow-up period was significantly higher in the pubertal patients than in the prepubertal ones (64.0 vs 34.3%; $\chi^2=5.7$, $P<0.01$), while no differences were found between boys and girls (40.0 vs 42.8%; $\chi^2=0.1$, $P>0.05$).

At the end of follow-up, the prevalences of pubertal patients in groups A (57.9%) and B (23.2%) were not significantly different when compared with the one found in group C (36.4%).

Overall, TSH values at the time of normalization did not significantly correlate with either the corresponding values of FT_4 or the time at normalization.

The prevalence of familial history of thyroid diseases was not significantly different in the patients who normalized TSH than in the ones with no TSH normalization (45.8 vs 54.2%; $\chi^2=1.6$, $P>0.05$).

Clinical and auxological data

None of the patients showed any clinical signs or symptoms of hypothyroidism during the entire follow-up.

During follow-up, no significant changes in the average *H* and BMI SDS were longitudinally observed in the entire study population and in the different groups as reported in Table 3. Both at entry and at the end of follow-up, the percentages of individuals with height deficiency were not significantly different in the entire series and in the three subgroups (Table 3).

In the overall study population, the percentage of individuals with overweight significantly decreased from entry to the end of follow-up, and this decrease was observed in both groups B ($\chi^2=5.5$, $P<0.01$) and A, although group A did not achieve a statistical relevance ($\chi^2=2.3$, $P>0.05$; Table 3). By contrast, in group C, the percentage of individuals with overweight remained stable from entry onward (Table 3). At the end of follow-up, the percentage of individuals with overweight was slightly higher in group C with respect to the one found in the remaining 81 patients (36.4 vs 16%), but this difference did not achieve a statistical relevance ($\chi^2=2.6$, $P>0.05$).

Data concerning the longitudinal evolution of pubertal stages, height, and BMI SDS during the entire follow-up in the patients of group C are analytically reported in Table 4. An evolution of pubertal stages throughout the 2-year follow-up was found in the four patients who had already entered puberty at the beginning of the study (nos. 4, 8, 9, and 10), while the remaining seven patients remained prepubertal during the entire follow-up (Table 4). The average height SDS at the end of follow-up did not significantly differ with respect to that recorded at study entry. In the two patients who exhibited a severe height deficiency at the end of study period, height deficiency was already present at entry (nos. 4 and 8, Table 4). Even BMI SDS did not significantly change from entry

Table 3 Average height (*H*) and BMI SDS and percentages of individuals with either severe height deficiency (<−2 SDS) or overweight (>90th percentile) at entry and at the end of follow-up in the overall study population and in the three subgroups with different TSH pattern during follow-up period.

	Overall (n=92)	Group A (n=38)	Group B (n=43)	Group C (n=11)
H at study entry	−0.1±1.3	0.1±1.2	−0.2±1.4	−0.7±1.2
H at study end	−0.1±1.3	−0.1±1.2	0.0±1.5	−0.9±1.1
BMI at study entry	0.6±1.7	1.1±1.5	0.3±1.8	0.8±1.7
BMI at study end	1.0±3.3	1.8±4.4	0.2±1.3	0.9±1.6
Patients with *H* deficiency at study entry (%)	8.7	2.6	11.6	18.2
Patients with *H* deficiency at study end (%)	3.3	0	2.3	18.2
Patients with overweight at study entry (%)	34.8	36.8	32.6	36.4
Patients with overweight at study end (%)	18.5	8.7	5.4	36.4

onward. Three patients were obese at study entry (nos. 2, 3, and 6, Table 4), but BMI did not change during follow-up.

Discussion

To the best of our knowledge, this is the first study aiming to prospectively investigate the spontaneous evolution over time of pituitary–thyroid function in a selected series of children and adolescents with idiopathic SH. With respect to the previous studies on young (3–7) or old patients (11–14), our study design is peculiar, in that we have preliminarily excluded all the underlying diseases and the risk factors that are known to be able to affect the natural course of SH (3–7).

According to our results, the natural history of TSH serum levels in a pediatric population with idiopathic SH is characterized by their progressive decrease over time, with no concomitant changes in FT_4 values. This 2-year prospective study shows that 38 patients (41.3%) out of a cohort of 92 patients under age 15 years normalized their TSH values during follow-up, and that only 11 of them (12%) underwent a deterioration of thyroid function, as demonstrated by an increase in TSH values in the range 10–15 mU/l. The risk of developing autoimmune thyroiditis in our series was unremarkable (2.2%).

Overall, this favorable evolution was more evident in the pubertal patients than in the prepubertal ones, while it was not significantly affected by either sex, or FT_4 levels or familial antecedents of thyroid diseases. In the overall study population, these changes in TSH serum concentrations over time were not associated with significant changes in clinical status and/or auxological parameters.

Our results as a whole allow us to confirm, on a larger series of patients, the conclusions by Moore (3) that juvenile SH is a benign and remitting process with a very low risk of evolution toward frank hypothyroidism. On the contrary, our results differ in some aspects from the ones previously reported in elderly patients with SH. In fact, Parle *et al.* investigated the evolution of 73 patients aged 60 years and found that 18% developed overt hypothyroidism and only 5% reverted to normal TSH values, although the follow-up period was only 12 months (13). Huber *et al.* studied a cohort of 82 women with a mean age of 50 years over a mean observation period of 9 years and found that 28% of them developed overt hypothyroidism, but only 4% became normal (11). The greater percentage of TSH normalization found in our survey may be explained by both the absence of previous history of either thyroid or non-thyroidal diseases and the only mild SH of all subjects who showed TSH values ranging between 5 and 10 mU/l. In fact, high baseline TSH levels, the presence of goiter, and positive thyroid autoantibodies have been demonstrated to be the main risk factors for the progression to thyroid failure (11, 13). It is not surprising, therefore, that a high percentage of reversion to normal TSH was also reported by Diez *et al.* who adopted a study design similar to the present one, by investigating only patients with mild thyroid hypofunction and no previous thyroid illnesses (2).

Table 4 Pubertal stages, height, and BMI both at entry and at the end of follow-up (lower panel) in the 11 patients of our series who exhibited TSH values >10 mU/l during follow-up (group C).

Patients	Pubertal stages	Height (SDS)	BMI (SDS)
At study entry			
1	B1P1	−0.06	−1.2
2	G1P1	0.2	2.7
3	B1P1	1.3	2.32
4	B3P2	−2.5	−1.9
5	G1P1	−1.8	−1.07
6	G1P1	0.28	3.35
7	G1P1	−0.08	0.88
8	G1P2	−2.2	1.1
9	G2P2	−1.4	0.9
10	G2P3	−0.28	1.34
11	B1P1	−1.0	0.5
Mean±s.d.		−0.7±1.2	0.8±1.7
At the end of follow-up			
1	B1P1	−0.5	−1.4
2	G1P1	−0.2	2.7
3	B1P1	1.0	2.3
4	B5P5	−2.5	−1.6
5	G1P1	−1.9	−0.6
6	G1P1	0.2	3.5
7	G1P1	−0.2	1.3
8	G4P4	−2.3	1.2
9	G5P5	−1.4	1.0
10	G5P5	−0.7	0.8
11	B1P1	−1.2	0.5
Mean±s.d.		−0.9±1.1	0.9±1.6

Nevertheless, it is to be underlined that in our series only a minority of patients (5.3%) reverted to TSH values lower than 2 mU/l, whereas most normalizers (68.4%) achieved TSH levels between 3 and 4.3 mU/l, a result very similar to the one recently reported by Diez *et al.* (12). The short duration of follow-up in the present study, however, does not allow us to predict the long-term evolution of these patients. In the early normalizers of the present study, TSH normalization was confirmed 12 months later, i.e., at the end of follow-up period. However, to further characterize the spontaneous evolution of SH in children, more prolonged follow-up studies are necessary.

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In conclusion, (a) the natural course of TSH values in a pediatric population with idiopathic SH is characterized by a progressive decrease over time; (b) the majority of patients (88%) normalized or maintained unchanged their TSH; (c) TSH changes were not associated with any changes in either FT_4 values or clinical status or auxological parameters; and (d) TSH determination has no reason to be part of the routine checkup in children, apart from specific protocols.

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CONCLUSIVE REMARKS

Thyroid hormones can have pleiotropic effects on multiple organ and tissues. The cardiovascular system is very sensitive to thyroid hormones and a wide spectrum of cardiovascular changes has long been recognized in overt and, more recently, in subclinical dysfunction. Our results document for the first time that young adults with congenital hypothyroidism may have abnormalities of cardiovascular system including an impairment of diastolic function, a reduction of exercise capacity and cardiopulmonary performance, increased IMT and impaired endothelial function. Such abnormalities occur despite careful replacement therapy and appear related to unphysiological fluctuations of TSH levels, with attendant episodes of subclinical hyperthyroidism and, more frequently, subclinical hypothyroidism. Thus, a cardiovascular follow-up should be performed, and future studies will clarify whether these abnormalities may result in clinically relevant cardiovascular diseases.

With regard to the prospective evaluation of children with subclinical hypothyroidism, we found that this condition in our group showed a progressive decrease over time and the majority of patients normalized or maintained unchainged their TSH values. Moreover, TSH changes were not associated with either FT4 values or clinical status or auxological parameters. A future perspective in this field will be to evaluate wether subclinical hypothyroidism in childhood is associated to increased incidence of markers of cardiovascular risk as well as in adulthood.

CHAPTER 6 COMPLEX INTERACTIONS BETWEEN ENDOCRINE AND OTHER BIOLOGICAL SYSTEMS: THE MODEL OF GROWTH HORMONE DEFICIENCY

6.1 Introduction and aims

The major function of GH in children is to promote linear growth, but GH has other important physiological effects which influence several key metabolic processes, including body composition, muscle strength, bone mineral density and reproductive capacity (1). Epidemiological studies suggest that adults with hypopituitarism have a reduced life expectancy compared to healthy controls, with increased mortality from cardiovascular disease even after thyroid, adrenal and hormone replacement (2;3). In fact, adults with untreated GH deficiency (GHD) may present important cardiovascular risk factors, such as hypercoagulability, abdominal obesity, insulin resistance, dyslipoproteinemia, reduced cardiac size and function and premature atherosclerosis with increased intima-media thickness (IMT) (4,5).

Recent data confirm that overall mortality and the rate of myocardial infarction are elevated in patients who have not undergone GH replacement (6). Untreated isolated GHD has also been reported to be associated with a significantly reduced life span. GH replacement exerts a beneficial effect on cardiovascular abnormalities by normalizing cardiac size and improving endothelial function, and on lipid profile in that it reduces fasting and postprandial lipoproteins and the atherogenic index. In addition, GH therapy decreases serum Hcy (7) and other inflammatory markers as well (8). These changes are likely to be beneficial in terms of cardiovascular risk.

Altogether, these results indicate that GH, directly or indirectly through IGF-I, is not only involved in the regulation of somatic growth in children but also in cardiac size and function, probably through the modulation of the size and function of myocardiocytes, endothelial structure and function, lipid profile and markers of inflammation.

Only a few studies have investigated cardiovascular risk in children affected by GHD. However, there is preliminary evidence that GHD also in children and adolescence may be associated with tith detrimental and cardiovascular and metabolic abnormalities such as reduced Left Ventricular (LV) mass (9), low flow-mediated endothelium dependent vasodilatation (10), abnormal lipid profile (11), increased serum Homocystein (Hcy) (7)

and inflammatory markers (12) which, although mild, may place them at higher risk of cardiovascular disease at an early age.

Aim of this phase of the project was to focus on metabolic and cardiovascular alterations associated to GH deficiency and evaluate whether these abnormalities are associated to GHD also in childhood.

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6.2 Improvement of cardiac performance and cardiovascular risk factors in children with GH deficiency after two years of GH replacement therapy: an observational, open, prospective,case-control study

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Improvement of Cardiac Performance and Cardiovascular Risk Factors in Children with GH Deficiency after Two Years of GH Replacement Therapy: An Observational, Open, Prospective, Case-Control Study

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Context: GH deficiency (GHD) in adults is associated with a cluster of cardiovascular risk factors that may contribute to an increased mortality for cardiovascular disease.

Objective: The aim of this study was to evaluate the effect of GHD and GH replacement therapy on cardiac performance, lipid profile, and insulin resistance in children.

Design: This was a 2-yr case-control prospective study.

Patients: Thirty children with GHD aged 9.3 ± 0.5 yr and 30 healthy matched controls were studied.

Intervention: Children were studied before and after 1 and 2 yr of GH replacement (GHD children) or no treatment (controls).

Main Outcome Measures: Lipid profile, serum insulin levels, homeostasis model of assessment (HOMA) index, and left ventricular (LV) mass and function by echocardiography were the main outcome measures.

Results: At study entry, the LV mass index was significantly lower in GHD children (50.2 ± 1.7) than in controls (60.3 ± 2.5 g/m^2; $P < 0.002$), whereas LV systolic and diastolic function, lipid profile, insulin levels, and HOMA index were similar. In GHD children LV mass index significantly increased (66.3 ± 2.4 g/m^2; $P < 0.0001$) after 1 yr of GH replacement and remained stable thereafter. LV systolic and diastolic function did not change during treatment. After 2 yr of GH replacement, total cholesterol ($P < 0.007$) and the atherogenic index ($P < 0.0001$) significantly decreased, whereas fasting insulin levels ($P < 0.001$) and HOMA index ($P < 0.0001$) significantly increased compared with both pretreatment and control values.

Conclusions: GHD in children is associated with a reduced cardiac size but with a normal cardiac function, lipid profile, and insulin sensitivity. Two years of GH replacement normalizes cardiac morphology, improves lipid profile, and slightly impairs insulin sensitivity. (***J Clin Endocrinol Metab* 91: 1288–1295, 2006**)

THE PRIMARY GOAL of GH replacement in children is to promote linear growth and to normalize final height to within or above the genetic target. GH has, however, other important physiological functions that influence several key metabolic processes, body composition, muscle strength, and bone mineral density. It is now established that adults with GH deficiency (GHD) may develop a cluster of cardiovascular risk factors, including unfavorable lipid profile, increased body fat, premature atherosclerosis, decreased fibrinolytic activity, increased peripheral insulin resistance (1, 2), as well as reduced cardiac performance (3, 4), all of which may contribute to a reduced life expectancy with an increased mortality for cardiovascular disease (CVD) (5).

In adolescents with severe GHD, there is increasing evidence that suggests that the discontinuation of GH replacement therapy at completion of linear growth may result in adverse effects on body composition, lipid profile, bone mineral density, cardiac morphology, and performance. However, there is still debate as to whether these abnormalities may predispose these patients to increased cardiovascular morbidity (6–9). The possibility that these changes may be a result of reversal of supraphysiological serum IGF-I due to the high GH doses used in some cases (6) should also be considered.

In contrast, relatively few studies have investigated whether or not children with GHD have metabolic and cardiac abnormalities that may place them at a higher risk of CVD at an early age. In the majority of these studies, lipoproteins in children with GHD are normal at baseline, but a beneficial effect of GH on lipid profile is observed during treatment (10–14). In addition, GH therapy reduces plasma

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Abbreviations: AI, Atherogenic index; BMI, body mass index; CVD, cardiovascular disease; DBP, diastolic blood pressure; E/A, ratio between maximal early diastolic flow velocity and maximal late diastolic flow velocity; FS, fractional shortening; GHD, GH deficiency; HDL, high-density lipoprotein; HOMA, homeostasis model of assessment; IRT, isovolumetric relaxation time; IST, interventricular septum thickness; LDL, low-density lipoprotein; LV, left ventricular; LVEDD, LV end-diastolic diameter; LVEDV, LV end-diastolic volume; LVEF, LV ejection fraction; LVESD, LV end-systolic diameter; LVESV, LV end-systolic volume; LVM, LV mass; LVMi, LV mass index; LVPWT, LV posterior wall thickness; SBP, systolic blood pressure; SDS, SD score.

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homocysteine levels, which are increased in children with GHD (14, 15). Elevated plasma homocysteine levels are considered to be independent risk factors for CVD.

Only a few studies have investigated the effect of GHD and GH replacement therapy on cardiac performance in children with GHD (16–18). GHD in children was associated with reduced cardiac mass, which increased after 1 yr of GH replacement therapy (17, 18). Conversely, neither GHD nor GH replacement was associated with alteration of cardiac function in children (17, 18).

The existing evidence indicates that atherosclerotic CVD begins in childhood (19, 20). In children, obesity occurs with other risk factors for CVD, such as increased blood pressure, adverse changes in serum lipoproteins, and hyperinsulinemia, leading to acceleration of atherosclerotic lesion; therefore, the primary prevention of atherosclerotic CVD should begin in childhood.

The aim of this observational, open, prospective, case-control study was to investigate the cardiovascular risk of GHD in prepubertal children. Therefore, cardiac mass and function, lipid profile, and degree of insulin resistance were evaluated in children with GHD before and after 1 and 2 yr of GH replacement therapy and in age-, sex-, pubertal status-, body surface area-, and body mass index (BMI)-matched controls before and 1 and 2 yr of observation. Moreover, to investigate whether the severity of GHD was also correlated with the degree of cardiac and metabolic impairment, children were divided in two groups on the basis of GH response at stimulation tests.

Patients and Methods

Patients

Thirty prepubertal children with GHD (18 boys and 12 girls) aged 9.3 ± 0.5 yr (range, 6.0–11.5 yr) were enrolled in the study. GHD was diagnosed according to clinical and auxological criteria (21) and by peak GH concentrations <10 μg/liter after two stimulation tests (mean peak GH after clonidine, 4.5 ± 0.5 μg/liter; after arginine, 3.5 ± 0.4 μg/liter). Twenty-seven children had isolated GHD; three had multiple pituitary hormone deficiency, and these children were receiving stable replacement with L-thyroxin, hydrocortisone, and 1-desamino-8-D-arginine vasopressin as necessary, before GHD was investigated. Magnetic resonance imaging of the hypothalamus-pituitary region documented pituitary hypoplasia in nine patients, ectopic posterior pituitary with stalk hypoplasia in five, empty sella in four, pituitary cyst in two, and craniopharyngioma in one. Before entry in the study, none of the patients received GH replacement. Previous or current CVD, respiratory, renal, or endocrine disease, or family history of CVD were exclusion criteria for entering the study.

Patients' profile at study entry is summarized in Table 1. Height and target height (sex-corrected midparental height) were expressed as SD score (SDS) according to the standards of Tanner (22).

Controls

Thirty healthy children (18 boys), age, sex, pubertal status, body surface area, BMI, socioeconomic status, and geographic area matched with the patients, were enrolled in the study as controls. As for the patients, previous or current CVD respiratory, renal, or endocrine disease, or history of CVD were exclusion criteria.

Study protocol

At study entry, all subjects underwent measurement of height, weight, heart rate, systolic (SBP) and diastolic (DBP) blood pressure, serum IGF-I, total cholesterol, high-density lipoprotein (HDL)-cholesterol, triglycerides, glucose and insulin levels, and echocardiography. We calculated the atherogenic index (AI) as the ratio of total/HDL cholesterol, considered as an index of severe cardiovascular risk (23). Low-density lipoprotein (LDL)-cholesterol was calculated using the Friedewald formula (24). Insulin resistance was evaluated by the homeostasis model assessment (HOMA) score, by applying the formula of Matthews *et al.* (25) [fasting serum insulin (microunits per milliliter) × fasting plasma glucose (millimoles per liter)/22.5].

In children with GHD, the evaluation of these parameters was also repeated after 1 and 2 yr of GH replacement therapy, whereas in controls evaluation was repeated after 1 and 2 yr of follow-up. Children with GHD were treated with a 30 μg/kg·d dose of GH (21).

To investigate whether the severity of GHD was correlated with the degree of cardiac impairment, children were divided in two groups on the basis of GH peak after stimulation tests. The group with severe GHD (n = 13) was characterized by a GH peak less than 5 μg/liter at both stimulation tests (range, 0.3–4.9 μg/liter); the group with partial GHD (n = 17) was characterized by a GH peak concentration above 5 μg/liter at one or both stimulation tests (range, 5.0- 9.5 μg/liter). Informed parental consent to participate in the study was obtained for both patients and controls.

Echocardiography

M-mode, two-dimensional, and pulsed Doppler echocardiographic studies were performed with ultrasound systems (Sonos 2000; Agilent Technologies, Andover, MS) using a 3.5-mHz transducer, during at least three consecutive cardiac cycles. The records were made by two investigators (L.S. and V.F.) blind in respect to the patients' status. All patients were studied in the left lateral recumbent position after a 10 min resting period according to the recommendations of the American Society of Echocardiography (26). The following measurements were recorded: interventricular septum thickness (IST), left ventricular (LV) posterior wall thickness (LVPWT), and LV end-systolic (LVESD) and end-diastolic (LVEDD) diameter; LV end-diastolic volume (LVEDV) and end-systolic volume (LVESV) were calculated according to the Simpson algorithm (27). The LV ejection fraction (LVEF) was calculated using the following formula: LVEF% = (LVEDV − LVESV)/LVEDV × 100. The fractional shortening (FS) percentage was calculated using the following formula:

TABLE 1. Clinical characteristics of the patients and controls at study entry

	GHD	Controls	*P*
No. of patients	30	30	NS
Sex (M/F)	18/12	18/12	NS
Chronological age (yr)	9.3 ± 0.5	9.8 ± 0.6	NS
Bone age (yr)	6.0 ± 0.5	8.0 ± 0.6	<0.02
Height (SDS)	−2.5 ± 0.3	−1.5 ± 0.2	<0.008
Target height (SDS)	−1.0 ± 0.1	−1.2 ± 0.1	NS
BMI (kg/m^2)	18.0 ± 0.8	17.2 ± 0.6	NS
Body surface area (m^2)	0.9 ± 0.04	1.0 ± 0.06	NS
IGF-I (SDS)	−2.0 ± 0.1	−0.1 ± 0.2	<0.0001
GH peak at arginine (μg/liter)	3.5 ± 0.4	16.2 ± 2.1	<0.0001
GH peak at clonidine (μg/liter)	4.5 ± 0.5		

Data are expressed as mean ± SE. NS, Not significant; M, male; F, female.

TABLE 2. Clinical, hormonal, and cardiac changes over 2 yr of GH replacement therapy in patients with GHD compared with controls

	Baseline	1 yr	2 yr	Repeated-measures ANOVA		Linear trend	
				F	P	F	P
Height (SDS)							
GHD	−2.5 ± 0.2[a]	−1.7 ± 0.2	−1.3 ± 0.3	25.53	0.0001	29.92	0.0001
Controls	−1.5 ± 0.1	−1.3 ± 0.2	−1.3 ± 0.2	2.63	0.096	5.41	0.03
IGF-I levels (SDS)							
GHD	−1.5 ± 0.1[a]	0.7 ± 0.3	0.6 ± 0.5	23.22	0.0001	17.29	0.001
Controls	0.03 ± 0.2	0.2 ± 0.3	0.1 ± 0.2	3.86	0.04	1.62	0.21
Heart rate (beats/min)							
GHD	81.9 ± 2.1	83.9 ± 1.9	80.1 ± 2.1	1.98	0.2	0.06	0.81
Controls	81.0 ± 2.8	80.9 ± 3.1	81.7 ± 3.3	0.79	0.46	0.05	0.83
SBP (mm Hg)							
GHD	90.2 ± 1.7	90.3 ± 2.5	95.3 ± 2.5	2.62	0.10	5.14	0.04
Controls	89.5 ± 3.0	93.1 ± 3.9	94.0 ± 4.1	3.06	0.097	3.59	0.09
DBP (mm Hg)							
GHD	59.0 ± 1.5	53.5 ± 1.9	53.3 ± 1.5	2.29	0.129	4.39	0.05
Controls	55.1 ± 2.5	54.0 ± 4.0	55.3 ± 3.9	0.08	0.93	0.06	0.82
IST (mm)							
GHD	5.9 ± 0.2	6.7 ± 0.2	7.3 ± 0.1	16.67	0.0001	29.28	0.0001
Controls	6.3 ± 0.3	6.6 ± 0.3	6.7 ± 0.2	3.31	0.06	6.8	0.02
LVPWT (mm)							
GHD	5.4 ± 0.2[b]	6.4 ± 0.2	6.8 ± 0.2	35.39	0.0001	39.28	0.0001
Controls	6.3 ± 0.2	6.6 ± 0.2	6.5 ± 0.2	2.18	0.15	64.69	0.05
LVESD (mm)							
GHD	22.0 ± 0.5	24.8 ± 0.9	25.6 ± 0.8	10.66	0.0001	21.06	0.0001
Controls	23.8 ± 0.9	25.5 ± 1.2	24.1 ± 0.6	1.65	0.23	0.33	0.57
LVEDD (mm)							
GHD	35.7 ± 0.7[b]	40.1 ± 0.9	40.3 ± 1.0	17.59	0.0001	20.33	0.0001
Controls	39.9 ± 1.1	40.9 ± 1.2	41.0 ± 1.1	0.68	0.52	0.17	0.69
LVMi (g/m^2)							
GHD	50.2 ± 1.7[b]	66.3 ± 2.4	68.1 ± 1.5	29.13	0.0001	55.10	0.0001
Controls	60.3 ± 2.5	63.8 ± 4.8	66.6 ± 3.0	4.92	0.02	8.51	0.01
FS (%)							
GHD	38.8 ± 0.8	38.1 ± 1.5	36.7 ± 0.7	1.81	0.185	3.68	0.07
Controls	40.8 ± 1.0	38.8 ± 1.3	38.6 ± 1.5	1.74	0.21	3.70	0.07
LVEF (%)							
GHD	65.2 ± 1.1	63.8 ± 1.1	64.9 ± 0.9	1.34	0.29	1.58	0.22
Controls	65.4 ± 1.2	66.4 ± 1.7	66.6 ± 1.3	1.82	0.20	3.05	0.10
IRT (msec)							
GHD	53.8 ± 2.0	51.5 ± 1.9	53.6 ± 1.3	0.90	0.43	0.44	0.51
Controls	57.9 ± 2.1	54.2 ± 3.3	55.6 ± 3.0	3.02	0.1	0.02	0.9
E/A							
GHD	1.8 ± 0.1	1.7 ± 0.08	1.8 ± 0.1	0.55	0.59	1.13	0.30
Controls	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	0.11	0.90	0.04	0.84

All values are expressed as the mean ± SE.
[a] $P < 0.0001$ *vs.* controls.
[b] $P < 0.002$ *vs.* controls.

whereas AI (3.7 ± 0.3 *vs.* 3.0 ± 0.1; $P < 0.03$) was significantly higher in children with severe than in those with partial GHD. A significant decrease in AI was observed during GH replacement in both children with severe GHD (to 3.0 ± 0.3 after 1 yr and to 2.6 ± 0.3 after 2 yr; $P < 0.01$) and partial GHD (to 2.5 ± 0.2 after 1 yr and to 2.3 ± 0.1 after 2 yr; $P < 0.0001$) (Fig. 2). At study entry, the HOMA index was comparable with controls (0.8 ± 0.3) in both severe GHD (0.8 ± 0.4) and partial GHD (0.6 ± 0.1) children. During GH treatment, the HOMA index increased more significantly in the group with partial GHD (to 1.4 ± 0.4 after 1 yr and to 2.2 ± 0.4 after 2 yr of GH; $P < 0.003$). After 2 yr of therapy, the HOMA index was higher in the group with partial GHD than in controls (0.9 ± 0.3; $P < 0.02$), although the increase was not statistically significant compared with children with severe GHD (1.3 ± 0.2; $P < 0.08$) (Fig. 3). At 2 yr, the dose of GH received by children with partial GHD (35 ± 0.8 μg/kg·d) was higher than that received by the group with severe GHD (30 ± 1.0 μg/kg·d; $P < 0.002$).

Discussion

A large number of studies have documented that adults (1–5) as well as adolescents (6–8, 29) with severe GHD have a cluster of cardiovascular risk factors that could place them at a higher risk of cardiovascular morbidity. It is now established that atherosclerotic CVD begins in childhood (19, 20). Relatively few studies, however, have investigated whether GHD in children is associated with increased risk factors for CVD. Shulman *et al.* (17) in a prospective, uncontrolled, study enrolling 10 children with GHD documented a reduced LVM that significantly increased after 1 yr of GH therapy, whereas cardiac function was not modified. The major drawback of the study by Shulman *et al.* was the lack

TABLE 3. Clinical and metabolic parameters in patients with GHD at baseline and over 2 yr of GH replacement therapy compared with controls

	Baseline	1 yr	2 yr	Repeated-measures ANOVA		Linear trend	
				F	P	F	P
BMI (kg/m^2)							
GHD	18.0 ± 0.8	18.8 ± 0.6	18.8 ± 0.8	1.55	0.25	2.48	0.14
Controls	17.2 ± 0.6	17.4 ± 0.8	18.1 ± 0.8	3.25	0.06	4.76	0.04
Triglycerides levels (g/liter)							
GHD	0.7 ± 0.04	0.7 ± 0.06	0.7 ± 0.06	0.205	0.18	0.38	0.54
Controls	0.6 ± 0.04	0.7 ± 0.2	0.6 ± 0.1	0.97	0.39	2.02	0.17
Total cholesterol levels (mmol/liter)							
GHD	4.1 ± 0.2	3.6 ± 0.1[a]	3.5 ± 0.1[b]	6.48	0.007	13.24	0.002
Controls	4.1 ± 0.1	4.1 ± 0.2	4.2 ± 0.1	2.08	0.15	0.79	0.38
LDL cholesterol levels (mmol/liter)							
GHD	2.5 ± 0.2	2.1 ± 0.2	2.1 ± 0.2	11.34	0.20	6.92	0.12
Controls	2.1 ± 0.3	2.0 ± 0.1	2.1 ± 0.1	1.80	0.19	0.001	0.98
HDL cholesterol levels (mmol/liter)							
GHD	1.4 ± 0.08	1.4 ± 0.08	1.5 ± 0.1	2.61	0.10	4.92	0.04
Controls	1.3 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	0.82	0.46	0.41	0.53
AI (total/HDL cholesterol)							
GHD	3.5 ± 0.4	2.6 ± 0.3	2.4 ± 0.2[a]	25.48	0.0001	53.23	0.0001
Controls	3.3 ± 0.4	3.1 ± 0.3	3.2 ± 0.3	0.66	0.52	0.10	0.75
Glucose levels (mg/dl)							
GHD	80.4 ± 1.9	85.0 ± 1.8	85.0 ± 2.1	0.93	0.42	0.28	0.60
Controls	81.1 ± 1.9	83.2 ± 2.0	84.0 ± 1.8	4.38	0.04	7.67	0.02
Insulin levels (mU/liter)							
GHD	4.4 ± 1.0	7.7 ± 1.2	9.2 ± 1.5[c]	11.93	0.001	25.34	0.0001
Controls	4.3 ± 1.2	4.2 ± 0.7	4.5 ± 0.8	2.74	0.1	1.8	0.19
HOMA index							
GHD	0.8 ± 0.2	1.7 ± 0.4	2.0 ± 0.3[c]	14.54	0.0001	30.05	0.0001
Controls	0.8 ± 0.3	0.8 ± 0.2	0.9 ± 0.3	1.9	0.20	4.30	0.07

Data are expressed as mean ± SE.
[a] $P < 0.03$ *vs.* controls.
[b] $P < 0.0001$ *vs.* controls.
[c] $P < 0.01$ *vs.* controls.

of a control group. In a previous short-term case-control study (18), we also demonstrated that heart size was significantly reduced in 12 GHD children and increased significantly after 1 yr of GH replacement.

The results of the current observational, open, prospective, case-control study further support the evidence of a significant reduction in cardiac size without changes of cardiac function in children with GHD. One year of GH replacement normalizes IGF-I levels and cardiac mass, and prolonged GH replacement for 2 yr does not further modify cardiac mor-

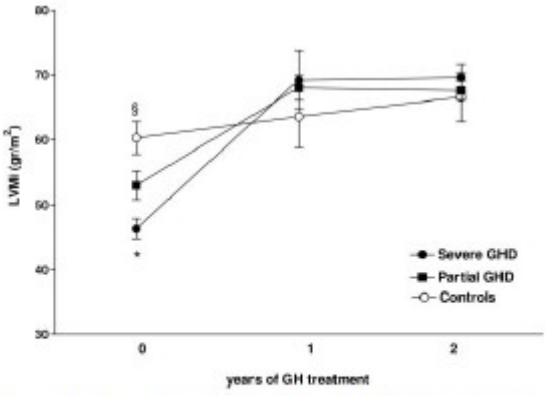

FIG. 1. Mean ± SEM measurements of the LVMi, before and during 2-yr GH therapy, in children with severe GHD compared with children with partial GHD and controls. *, $P < 0.03$ comparing children with severe *vs.* partial GHD at study entry. §, $P < 0.005$ comparing both children with severe and partial GHD *vs.* controls at study entry.

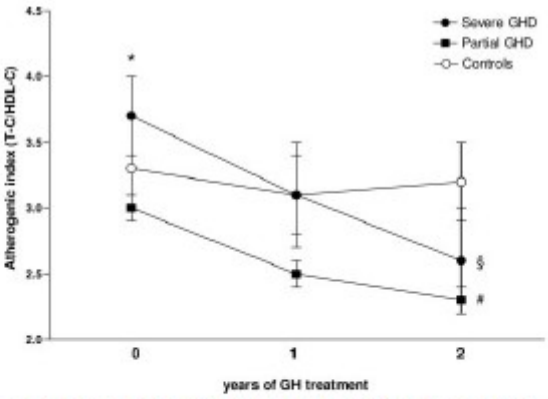

FIG. 2. Mean ± SEM values of AI in children with severe GHD compared with children with partial GHD and controls, before and during 2-yr GH therapy. *, $P < 0.03$ comparing children with severe *vs.* partial GHD at study entry. §, $P < 0.01$ analyzing the effect of GH treatment during the study in the group with severe GHD. #, $P < 0.0001$ analyzing the effect of GH treatment in the group with partial GHD.

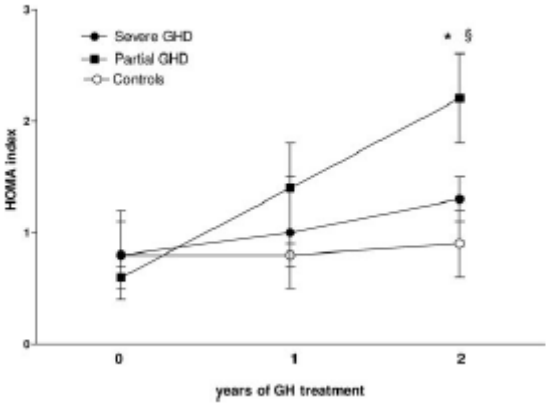

FIG. 3. Mean ± SEM values of the HOMA index in children with severe GHD compared with children with partial GHD and controls before and during 2 yr of GH therapy. *, $P < 0.003$ analyzing the effect of GH treatment during the study in the group with partial GHD. §, $P < 0.02$ comparing children with partial GHD *vs.* controls after 2 yr of follow-up.

phology and function. Therefore, replacement GH treatment, in the dose of 30 μg/kg·d, does not have any hypertrophic effect on the heart.

In a previous study on 14 adolescents with partial GHD, Radetti *et al.* (16) reported that LVM and systolic and diastolic functions did not differ from a control group after 1 yr of GH therapy at a relatively high dose of GH (44 μg/kg·d). After 5 yr of GH treatment, however, an increase in LVM and a mild impairment in LV diastolic function was observed (16). The major drawback of that study was the lack of baseline data. The different results with respect to the present study may depend on different GH dosages, duration of GH treatment, and degree of GHD. In particular, the different degree of GHD may result in a different response to GH replacement, which might ultimately result in a varied increase in cardiac size. The results of the present study indicate that children with severe GHD have a more evident reduction in LVM than children with partial GHD, although in both groups LVM was significantly reduced compared with controls. Using a standard dose of GH, we did not observe any different response between the two groups. After 2 yr of GH replacement, severe GHD, partial GHD, and controls had similar cardiac size. Altogether, these results indicate that GH, directly or indirectly through IGF-I, is not only involved in the regulation of somatic growth in children but also in cardiac growth, probably through the modulation of the size of cardiomyocytes (30).

We did not observe significant gender-related differences on cardiac mass and function both at baseline and in the long-term effects of GH replacement therapy.

Several studies have investigated plasma lipoproteins in children with GHD, providing conflicting results (10, 11, 13–15). In adults with untreated GHD and in adolescents with severe childhood-onset GHD at discontinuation of GH, the most common lipid pattern is represented by increased total and LDL cholesterol and decreased HDL cholesterol levels, increased triglycerides, and AI (1, 7, 8, 31). Conversely, the majority of the studies in children with GHD failed to find abnormalities in the lipid profile at baseline (10, 11, 13–15). The difference between adults and children with GHD may reflect the population trend for a rise in cholesterol and LDL cholesterol with increasing age.

In the present study, as in our previous studies (14, 18), we did not find any difference in lipid profile between GHD children and controls at baseline. During 2 yr of GH treatment, we observed an improvement in lipid profile, with a significant decrease in total cholesterol and in AI compared with both the baseline and the control levels. In agreement with others (12), we did not observe significant gender-related differences in the long-term effects of GH replacement therapy.

This beneficial effect of GH treatment on AI has been reported in other short- and long-term studies evaluating the efficacy of GH therapy on lipid profile in GHD children (10–12, 14). In a 6 yr follow-up study, van der Sluis *et al.* (13) documented a long-term beneficial effect of GH therapy on AI, as well as on HDL cholesterol in GHD children. It is well known that abnormalities in lipid profile may severely increase the coronary risk of GHD patients (32); thus, the decrease in the total/HDL cholesterol ratio during GH therapy can be clinically relevant to the prevention of CVD in midlife, because it represents one of the most efficient predictors of coronary heart disease in adults (33).

The exact mechanisms that underlie these changes are not fully understood, but GH may act through the regulation of both the activity of the cholesterol 7 α-hydroxylase enzyme and the regulation of LDL cholesterol receptor numbers (34). However, other mechanisms are likely to be involved and require additional investigation.

Concern has been expressed that GH administration in children and adolescents may cause or exacerbate, in predisposed individuals, type 2 diabetes mellitus (35). The effect of GH treatment in adults with GHD on glucose metabolism is still a matter of debate. Most short-term studies have reported a deterioration of insulin sensitivity, whereas long-term studies suggested that, after an initial worsening, insulin sensitivity returned toward baseline values (36). Children with GHD do not have insulin resistance at baseline; on the contrary, they have in infancy a tendency toward fasting hypoglycemia, whereas susceptibility to hypoglycemia diminishes with age and paradoxically GH-deficient adults may show insulin resistance even before GH replacement therapy. The effect of GH therapy on glucose metabolism in children with GHD has not been extensively investigated. Previous studies in short children have shown that short-term GH replacement was associated with development of insulin resistance and peripheral hyperinsulinemia, as measured by the hyperglycemic clamp technique or using oral glucose tolerance testing, even if insulin levels remained within the physiological range of normal control children (37, 38). In short small-for-gestational-age children, GH replacement induces high fasting insulin levels with normal glucose levels, suggesting insulin resistance in these patients. However, 6 months after GH discontinuation, insulin levels returned to normal values compared with a control group (39). No case of impaired glucose tolerance or diabetes was re-

corded in a large group of 128 GHD children treated with GH for a period of 6 yr, but a significant decrease in insulin sensitivity was detected during the first year of GH therapy (40).

In the present study, we observed a mild increase in insulin resistance after 2 yr of GH treatment, especially in children with partial GHD who were receiving a slightly higher dose of GH. However, additional follow-up is necessary to evaluate whether insulin sensitivity will continue to worsen as an effect of GH therapy, or this mild increase may instead represent a component of the anabolic process of somatic development as is clearly evident in puberty.

In conclusion, GHD in children is associated with a significantly reduced cardiac size and with a normal cardiac function. Two years of GH replacement normalizes cardiac morphology and does not modify cardiac function. GHD is not associated with a clear-cut impairment of lipid profile, but 2 yr of GH replacement therapy exerts a beneficial effect on it by reducing total cholesterol and AI. On the contrary, a trend toward an increase in insulin resistance is observed during GH treatment. The potential long-term negative effect of insulin resistance determined by GH replacement on cardiovascular morbidity is still to be determined.

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The authors have nothing to declare.

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6.3 REVIEW: METABOLIC EFFECTS OF GH DEFICIENCY AND GH REPLACEMENT THERAPY IN CHILDREN AND ADOLESCENTS.

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Abstract

Adults with severe GH deficiency (GHD) may develop a cluster of cardiovascular risk factors that may contribute to a reduced life expectancy with an increased mortality due to cardiovascular disease. Also in adolescents with severe GHD there is increasing evidence which suggests that the discontinuation of GH replacement therapy at completion of linear growth may result in adverse effects on body composition, lipid profile, cardiac morphology and performance. In contrast, relatively few studies have investigated whether or not children with GH deficiency have metabolic and cardiac abnormalities that may place them at a higher risk of cardiovascular disease at an early age. This review focuses on the effect of both GH deficiency and GH replacement on cardiovascular risk factors in children and adolescents with GHD.

Introduction

The major role of GH treatment in children and adolescents with GH deficiency (GHD) is to promote linear growth and to normalize final height to within or above the genetic target. In adults severe GHD has been shown to be associated with reduced quality of life, decrease bone mineral density, reduced muscle mass, increased fat mass and with several metabolic abnormalities contributing to increased cardiovascular risk [1-3]

Epidemiological data suggest that adults with hypopituitarism who are on conventional thyroid, adrenal and gonadal hormone replacement, but not GH, have a reduced life expectancy with a twofold increase in mortality from cardiovascular disease [4,5]. The markers of cardiovascular risk associated with untreated GHD include unfavorable lipid profile, increased body fat, decreased fibrinolytic activity, increased peripheral insulin resistance, reduced cardiac size and function, premature atherosclerosis with increased carotid intima-media thickness [6,7].

In adolescents with severe GHD, the discontinuation of GH replacement therapy at completion of linear growth may result in adverse effects on body composition, lipid profile, cardiac morphology<and performance [8-10].

Atherosclerosis begins to appear with greater frequency in childhood and young adulthood and the extent of atherosclerotic lesions increases markedly in young people with multiple risk factors [11].

Relatively few studies have investigated whether GH deficiency in children may have cardiovascular and metabolic effects which may place these patients at a higher risk of cardiovascular disease at an early age. As such, the aim of this article is to review the effect of both GH deficiency and GH replacement in children and adolescents.

Metabolic effects of GHD and GH replacement during the transition phase

The appropriate management of GHD patients during transition from childhood to adulthood is still being debated. Discontinuation of GH therapy for 1 year in adolescents with severe GHD results in the accumulation of cardiovascular risk factors such as increase of total body and abdominal fat, decrease of lean body mass, and increase of total cholesterol (TC) with a decrease in high-density lipoprotein cholesterol (HDL-C) [8]. Moreover, Colao et al [10] have shown that in severe GHD adolescents, triglycerides, low-density lipoprotein cholesterol (LDL-C), the total/HDL-C ratio and fibrinogen levels significantly increase six months after GH withdrawal and return to the levels measured before GH cessation six months after restarting GH replacement. In addition, GH discontinuation remarkably reduces IGF-I levels and modifies heart morphology and function. Using echocardiography, left ventricular mass index (LVMi) decreases significantly 6 months after GH withdrawal and significantly improves 6 months after GH resumption. Systolic function, measured as LV ejection fraction (LVEF) is not significantly modified, however a trend toward impairment at GH discontinuation and improvement after GH resumption can be observed. The ratio between the maximal early (E) and maximal late (A) diastolic flow velocity (an index of ventricular filling) is also significantly decreased by GH withdrawal. Other studies did not find any striking changes either with continuation or cessation of GH replacement in adolescents with severe GHD [12,13]. No significant changes in body composition,

LV mass and function, lipids and carbohydrate metabolism were observed in adolescents with severe GHD treated with GH compared to controls and those treated with placebo during a 2-year double blind, placebo-controlled study [12]. Continuation of GH treatment was associated with gain in lean body mass (LBM), but no significant change was found during a year of observing adolescents with severe GHD who had ceased GH treatment [13]. Conversely, Hulthen et al [14] demonstrated that the increase in

LBM and muscle performance observed in healthy adolescents between the age of 17 and 21 did not occur in adolescents with GHD when GH treatment was discontinued for 2 years. Patients with childhood-onset or adult-onset GHD have been shown to have an increased number of atheromatous plaques in the carotid and femoral arteries, with increased intima-media thickening (IMT) of the carotid arteries, increased stiffness of the carotid wall and impaired flow-mediated endothelium-dependent dilation of the brachial artery [15,16]. To investigate the risk of early atherosclerosis, Colao et al [17] measured IMT at the common carotid arteries in GHD adolescents during GH replacement and withdrawal. Before GH discontinuation, IMT at common carotid arteries in adolescents with severe GHD was similar to age and gender matched controls and did not change 6 months after GH withdrawal or 6 months after GH treatment was resumed, thus suggesting that short-term withdrawal of GH replacement is not associated with vascular abnormalities during the transition phase.

The management of adolescents with GHD during the transition phase in terms of GH replacement remains a difficult challenge. It seems, however, that GH withdrawal in patients with reconfirmed GHD may lead to several unfavorable changes that can be reversed by restarting GH treatment.

Metabolic effects of GHD and GH replacement during in childhood and early adolescence

In adults with GHD, cardiovascular and metabolic alterations have been extensively documented, while metabolic changes in GHD children and adolescents have only recently begun to be investigated. Some studies have shown that serum levels of TC, LDL-C, Lp (a), apolipoprotein B (ApoB) [18,19] and triglycerides (TG) [20] are raised in untreated GHD children and adolescents when compared to both GH treated adolescents and healthy controls. Conversely, in other studies no striking abnormalities in lipid profile were detected at baseline in children with GHD [21-27]; however TC, LDL-C, and the atherogenic index were above the upper limits in several untreated GHD patients [21,25,27].

In the majority of studies, both short and long-term GH replacement therapy in GHD children induced a beneficial effect on lipid profile [21-24,27]. In our study on 30 children with GHD [22] no significant differences were observed at baseline between GHD children and healthy controls; however, 2 years of GH replacement induced a

significant decrease in total-cholesterol and the atherogenic index compared to both pre-treatment and control values. The decrease in the atherogenic index was significantly correlated with the increase in IGF-I levels. Similar results were also reported by Van der Sluis et al [27] in a 6-year follow-up study. During GH replacement therapy, the atherogenic index and LDL- cholesterol decreased significantly and HDL- cholesterol improved [27]. Beside the atherogenic index, which represents one of the most reliable predictors of coronary heart disease in adults [28], postprandial TG and TG-rich lipoprotein particles, have been found to play a role in the atherogenic process. Abnormal postprandial lipids have been reported in adults with GHD [29] and in untreated GHD adolescents as well [20] however GH therapy significantly improve both the fasting and the postprandial atherogenic lipoprotein profile [20,30]. The exact mechanisms that underlie lipoproteins abnormalities are not fully understood. The expression of several hepatic surface receptors such as LDL and LDL-related protein receptors is lower in GH deficiency and increases with GH treatment [31]. Thus, the accumulation of atherogenic lipoproteins may be the result of a decrease in their removal from circulation via hepatic lipoprotein receptors. Inflammation as well plays an essential role in the initiation and progression of atherosclerotic lesions. Elevated levels of inflammatory markers such as C- reactive protein (CRP), interleukin-6 (IL-6), fibrinogen, homocysteine (Hcy), have been found to be associated with increased cardiovascular risk [32]. Plasma levels of inflammatory markers such as IL-6 and TNF-□, are increased during the postprandial period and are related to the presence of elevated levels of lipoprotein remnants, suggesting that these lipoproteins may induce an inflammatory response in endothelial cells and macrophages through specific receptors on their surface [31].

In untreated adolescents with GHD, fasting CRP, TNF-□ and fibrinogen concentrations were higher compared to healthy control, but similar to those of patients treated with GH; moreover, fasting and postprandial TG of untreated adolescents with GHD were positively associated with fasting and postprandial CRP levels and with postprandial TNF-□ and IL-6 concentrations [30]. Fasting fibrinogen levels were elevated in both treated and untreated adolescents with GHD when compared to controls, and were positively correlated with fasting TG levels [30]. No differences were observed as concerned PAI-1 concentrations [20]. Conversely, abnormalities in coagulation factors suggestive of a defective fibrinolytic system have been reported in adults with GHD with beneficial effects of GH replacement on fibrinogen levels [33], on PAI-1 activity, PAI-1 antigen and tissue plasminogen activator (t-PA) antigen levels [34]. Thus,

adolescents with GHD seem to have a pronounced inflammatory response but the role of GH treatment in the reduction of inflammatory markers seems less clear since CRP, TNF-□ and fibrinogen were elevated in both treated and untreated GHD adolescents. Elevated plasma Hcy levels are actually considered to be an independent risk factor for atherosclerosis and thromboembolism and thus also for cardiovascular disease [3]. In children moderate hyperhomocisteinemia is related to an increased risk of ischemic stroke [35]. The mechanism of Hcy action on the cardiovascular system is not still completely understood, however, elevated plasma Hcy levels increase collagen production, decrease the availability of NO and have a direct endotelial cytotoxicity [36].

In a cross-sectional study, plasma Hcy levels were found to be elevated in untreated GHD adolescents compared to both treated GHD and controls [20]. We confirmed these findings in a longitudinal prospective study [26] on prepubertal children with GHD. At study entry plasma Hcy levels were significantly higher than in healthy age and sex matched children, although the absolute values were within normal range. After 1 year of GH replacement therapy, Hcy levels decreased significantly, reaching values comparable to controls. In addition to dyslipidemia, inflammation and a prothrombotic state, an increased IMT of both common carotid arteries represents one of the earliest morphological changes in the arterial wall in the process of atherogenesis; it is also an independent predictor of acute myocardial infarction in men [3]. It is now established that silent atherosclerotic disease with atheromatous changes in blood vessels begins to develop during childhood and progresses with age [37].

The GH/IGF-I axis also plays an important role in vascular disease. Endothelial cells possess receptors for IGF-I and circulating IGF-I levels have a direct stimulatory effect on endothelial properties through induction of nitric oxide (NO). A decrease in NO activity in GHD patients is associated with impaired arterial vasodilator capacity, increased platelet aggregability and intimal thickening [6]. In adults with GHD short and long-term GH replacement therapy has been recognized to have either an inhibitory effect on IMT progression or induce a significant improvement of the vasodilatory function of the endothelium [3].

Carotid artery IMT measurements were significantly increased in 25 untreated children and adolescents with GHD compared to healthy controls [19]. Conversely, in a recent study, Lanes et al [38] were unable to find a significant increase in the IMT of the carotid arteries in untreated GHD adolescents when compared with that of those treated with GH and controls, however in the GHtreated group, a trend toward a decrease in IMT

values could be observed. Moreover, the flowmediated endothelium-dependent vasodilatation was lower in untreated GHD adolescents than in GHD treated and control groups. Thus, an improvement in endothelial function and a reduction in arterial stiffness appears to occur after GH replacement. In addition, the epicardial adipose tissue on the right ventricle, which seems to be a good marker for increased cardiovascular risk in adults, is significantly increased in untreated GHD adolescents as measured by echocardiography, whereas in the GHD treated group the epicardial adipose tissue is comparable to controls [38]. Finally, GHD in children affects heart morphology by inducing a significant decrease in cardiac size without modifying cardiac function [21,22,38,39]. Clinical and experimental studies have shown that GH and IGF-I are involved in the regulation of heart function and morphology [6,7]. Moreover, IGF-I directly causes cardiac hypertrophy of cultured rat cardiomyocytes, increases myocardial contractility by enhancing the calcium sensitivity of myofilaments in cardiomyocytes and delays cardiomyocytes apoptosis. In our study [22], a group of 30 children with GHD and another group of 30 healthy children matched by age, sex, pubertal status, body surface area, body mass index (BMI), were each studied before and after 1 and 2 years of GH replacement (at a mean dose of 30 □g/kg/day) or no treatment. Heart rate, systolic and diastolic blood pressure were normal at study entry and remained unchanged throughout years 1 and 2 of GH therapy. Echocardiography showed that LV posterior wall thickness (LVPWT), LV end-diastolic diameter (LVEDD), and LVMi were significantly reduced in GHD children before GH replacement therapy compared to controls, and significantly increased during the first year of GH therapy compared to pre-treatment values. They remained unchanged during the second year of GH replacement. The increase in LVMi was significantly correlated with the increase of IGF-I levels. LV systolic function and diastolic function were similar in GHD children and control subjects at baseline and did not significantly change during 2 years of treatment [22]. Conversely, in a group of children and adolescents with partial GHD, 5 years of GH therapy at a relatively high dose of GH (44 □g/kg/day) resulted in an increase in LV mass and a mild impairment in LV diastolic function, as evaluated by 2-dimensional M-mode echocardiography, thus indicating a sub-clinical morpho-functional alteration of the left ventricle in some of them after long-term GH treatment with relatively large doses [40].

CONCLUSIONS

Taken as a whole these results indicate that GH deficiency in children and adolescents may be associated with detrimental cardiovascular and metabolic abnormalities such as abnormal lipid profile, increased serum inflammatory markers, reduced LV mass and a mild endothelial dysfunction. GH replacement therapy exerts a beneficial effect on cardiovascular abnormalities, on lipid profile, and serum Hcy as well. These changes are likely to be beneficial in terms of cardiovascular risk, however, long-term clinical studies are necessary to definitively clarify these issues. Meanwhile the evaluation of cardiovascular and metabolic changes should be added to the management of GHD patients during childhood and adolescence.

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6.4 Subtle alterations of cardiac performance in children with GH deficiency: results of a two years prospective, case-control study

ORIGINAL ARTICLE

Endocrine Care

Subtle Alterations of Cardiac Performance in Children with Growth Hormone Deficiency: Results of a Two-Year Prospective, Case-Control Study

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Background: GH-deficient (GHD) children have reduced left ventricular (LV) mass, but impairment of cardiac function has never been documented.

Aim: The aim of the study was to evaluate effects of GHD and GH therapy on cardiac function using load-dependent and load-independent indices of myocardial contractility.

Patients and Methods: Echocardiography was performed in 24 GHD children at baseline and 1 and 2 yr after GH therapy and in 24 controls.

Results: Compared with controls, GHD children at baseline had lower LV mass (LV mass/BSA 50.6 ± 1.8 *vs.* 60.5 ± 2.4 g/m^2; $P < 0.002$, and LV mass/H$^{2.7}$ 28.7 ± 1.2 *vs.* 33.6 ± 1.3 g/m$^{2.7}$; $P < 0.009$). Global systolic function was normal, with only a trend toward slight impairment of the fractional shortening (34.9 ± 1.5 *vs.* 37.6 ± 1.1%). However, subtle LV dysfunction was revealed by load-dependent and load-independent indices of myocardial contractility. In fact, GHD patients compared with controls showed lower rate-corrected mean velocity of circumferential fiber shortening (1.0 ± 0.03 *vs.* 1.18 ± 0.03 circ/sec; $P = 0.0001$) and stress shortening index (0.10 ± 0.02 *vs.* 0.18 ± 0.02; $P < 0.007$) and higher end-systolic stress (49.2 ± 1.4 *vs.* 45.7 ± 1.0 g/cm^2; $P < 0.05$). One year of GH treatment was associated with a significant improvement of cardiac size (LV mass/BSA 67.8 ± 2.9 g/m^2; LV mass/H$^{2.7}$ 38.2 ± 2.0 g/m$^{2.7}$; $P < 0.0001$ and $P = 0.0003$, respectively) and myocardial contractility (mean velocity of circumferential fiber shortening 1.2 ± 0.04 circ/sec; $P < 0.0002$; stress shortening index 0.19 ± 0.02; $P < 0.003$) and reduced afterload (end-systolic stress 43.9 ± 1.4 g/cm^2; $P < 0.03$).

Conclusions: Our data indicate that GH deficiency is associated with abnormalities in morphology and function in not only adults but also children and further supports the beneficial effect of GH on the heart. (*J Clin Endocrinol Metab* 94: 3347–3355, 2009)

Even if the primary goal of GH replacement therapy is to promote linear growth in children, it has now been established that GH also has other important physiological functions influencing several key metabolic processes. Among the distinct features of GH deficiency (GHD), cardiovascular involvement has emerged as particularly important. Previous studies on GHD and GH therapy have documented that adults as well as adolescents with severe

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Abbreviations: AI, Atherogenic index; BSA, body surface area; CVD, cardiovascular disease; DBP, diastolic blood pressure; E/A, maximal early to late diastolic flow velocity ratio; ESS, end-systolic wall stress; FS, fractional shortening; GHD, GH deficiency; HDL, high-density lipoprotein; IRMA, immunoradiometric assay; IRT, isovolumetric relaxation time; IVST, interventricular septum thickness; LDL, low-density lipoprotein; LV, left ventricular; LVEDD, left ventricular end diastolic diameter; LVEF, left ventricular ejection fraction; LVEDV, left ventricular end diastolic volume; LVESD, left ventricular end systolic diameter; LVESV, left ventricular end systolic volume; LV mass/BSA, left ventricular mass corrected for BSA; LV mass/H$^{2.7}$, left ventricular mass corrected for height × 2.7; LVPWT, left ventricular posterior wall thickness; mVCFc, mean velocity of circumferential fiber shortening; SBP, systolic blood pressure; SDS, SD score; SSI, stress shortening index.

GHD have a cluster of cardiovascular risk factors, including increased risk of atherosclerotic or thrombotic disease with impaired lipid profile, vascular function, and homocysteine, as well as impairment of cardiac morphology and function, that could place them at a higher risk of cardiovascular morbidity (1–4).

GHD in children affects heart morphology by inducing a significant decrease in cardiac size (5–8). Echocardiography has revealed that left ventricular (LV) mass index is significantly reduced in GHD children before GH therapy compared with controls and significantly increased during the first year of therapy (4, 6, 8). Neither GHD nor GH therapy has ever been associated with a significant impairment of cardiac function.

The effect of GHD and GH therapy on cardiac performance have yielded heterogeneous results in adults as well. Studies using radionuclide angiography have demonstrated that adults with GHD have impaired cardiac performance, especially at peak exercise (9–11), whereas echocardiographic studies have shown contrasting results (12).

It should be noted that echocardiography might not be sensitive enough to reveal minimal abnormalities in cardiac performance. In fact, LV ejection fraction (LVEF) and fractional shortening (FS), the most widely used indices for evaluating systolic function, may not be able to detect subclinical dysfunctions, because they are highly dependent on and influenced by myocardial loading.

Ideally, to discriminate between inherent ventricular dysfunction and the effects of altered loading conditions, myocardial contractility in children should also be evaluated using load-independent indices of ventricular function. Indeed, to detect subclinical myocardial alterations, the evaluation of the rate-corrected mean velocity of circumferential fiber shortening (mVCFc), the end-systolic wall stress (ESS), and their relationship as determinants of systolic performance can be used.

We designed this observational, open, case-control, prospective study to investigate systolic function in prepubertal GHD children before and after 1 and 2 yr of GH therapy by using load-dependent and load-independent indices of myocardial contractility.

We also evaluated whether the severity of GHD was correlated with the degree of LV systolic impairment as well as whether there were gender differences of GHD and GH therapy on cardiac performance.

Patients and Methods

Twenty-four prepubertal children with GHD (12 boys and 12 girls) aged 8.8 ± 0.4 (range 4.2–13.1 yr) were enrolled. GHD was diagnosed according to clinical and auxological criteria (13) and by peak concentration less than 10 μg/liter after two stimulation tests. Twenty-one children had isolated GHD; three children with multiple pituitary hormone deficiency were receiving stable replacement therapy with L-thyroxine, hydrocortisone, and 1-desamino-8-D-arginine vasopressin as necessary. Magnetic resonance imaging of the hypothalamus-pituitary region documented pituitary hypoplasia in five patients, ectopic posterior pituitary with pituitary hypoplasia in two, pituitary cyst in one, ectopic posterior pituitary in one, and empty sella in two. Before entry in the study, none of the patients had ever received GH replacement. Previous or current cardiovascular disease (CVD), respiratory, renal, or endocrine disease or family history of CVD were criteria for exclusion from the study. None of the patients entered puberty during the first year of therapy. Clinical details of the patients at study entry are summarized in Table 1.

TABLE 1. Clinical details of the patients and controls at study entry

	GHD	Controls	P
No. of patients	24	24	NS
Sex (male/female)	12/12	12/12	NS
Chronological age (yr)	8.8 ± 0.4	7.5 ± 0.5	<0.05
Bone age (yr)	6.6 ± 0.5	NA	
Height (SDS)	−2.3 ± 0.3	−1.2 ± 0.2	<0.004
Height (cm)	117 ± 2.6	118 ± 2.9	NS
BSA (m^2)	0.9 ± 0.04	0.88 ± 0.04	NS
Body mass index (kg/m^2)	17.7 ± 0.8	16.5 ± 0.6	NS
IGF-I (SDS)	−1.2 ± 0.1	0.1 ± 0.2	<0.0001
GH peak after arginine (μg/liter)	3.8 ± 0.09	NA	
GH peak after clonidine (μg/liter)	5.2 ± 0.6	NA	

Data are expressed as means ± SE. NS, Not Significant; NA, not available.

Controls

Twenty-four healthy children, which were sex, pubertal status, height, body surface area (BSA), socioeconomic status, and geographic area matched with the patients, were enrolled in the study as controls (Table 1). These subjects were referred to our unit for short stature and after a complete evaluation including GH stimulation tests were diagnosed as having familial short stature. Height was expressed in both centimeters and SD score (SDS) according to the Tanner standards (14). Because children with GHD may have abnormal body composition, with a reduction in lean mass and an increase in body fat, we compared the patients with a group of controls who were younger but height and BSA matched to better normalize cardiac parameters. Previous or current CVD, respiratory, renal, or endocrine disease, or history of CVD were exclusion criteria for the patients.

Study protocol

At study entry, all subjects underwent measurement of height, weight, BSA, heart rate, systolic (SBP) and diastolic (DBP) blood pressure, serum IGF-I, and echocardiography.

In children with GHD, the evaluation of these parameters was also repeated after 1 and 2 yr of GH therapy, whereas in controls evaluation was repeated after 1 and 2 yr of follow-up. Children with GHD were treated with a 30 μg/kg · d dose of GH (13).

To investigate whether the severity of GHD was also correlated with the degree of cardiac impairment, children were divided into two groups on the basis of GH peak after stimulations. The group with severe GHD (n = 11) was characterized by a GH peak of less than 5 μg/liter at both stimulation tests; the group

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with partial GHD (n = 13) was characterized by a GH peak concentration above 5 μg/liter at one or both tests. We also evaluated the severity of GHD on the basis of IGF-I levels, subdividing patients into two groups: the group with severe GHD consisting of those patients who had an IGF-I below (n = 11) and the group with partial GHD of those patients with an IGF-I above (n = 13) − 1.5 SDS at diagnosis.

Moreover, fasting lipids and homocysteine were also evaluated before and during GH therapy as indices of atherosclerotic or thrombotic risk, respectively. Informed parental consent for participation in the study was obtained for patients and controls.

Assays

Serum GH levels were measured by immunoradiometric assay (IRMA) using a commercially available kit (HGH-CTK-IRMA; Sorin, Saluggia, Italy).

Serum IGF-I levels were measured using a two-site IRMA kit (Diagnostics System Laboratories, Inc., Webster, TX). The IGF-I intra- and interassay coefficients of variation were 3.4 and 8.2%, respectively. The normal range was 60–350 μg/liter in children aged younger than 5 yr and 180–780 μg/liter in adolescents aged 11–18 yr.

Total and high-density lipoprotein (HDL) cholesterol, triglycerides, and homocysteine were measured by standard procedures. We also calculated the atherogenic index (AI) as the ratio of total to HDL cholesterol, considered as an index of severe cardiovascular risk (15). Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula (16).

Echocardiography

M-mode, two-dimensional, and pulsed Doppler echocardiographic studies were performed with an ultrasound system (Acuson 128 XP/5; Acuson, Mountain View, CA) using 3.5- and 5-mHz transducers, during at least three consecutive cardiac cycles. The records were made by two investigators (V.F., L.S.) blinded to the subjects' status. All patients were studied according to the recommendations of the American Society of Echocardiography (17).

Cardiac morphology was evaluated by the following measures: interventricular septum thickness (IVST), LV posterior wall thickness (LVPWT), LV end-systolic (LVESD), and end-diastolic (LVEDD) diameters. LV end-diastolic volume (LVEDV) and end-systolic volume (LVESV) were calculated according to the Simpson algorithm (18). LV mass was calculated using the Devereux formula according to Penn's convention with the regression-corrected cube formula LV mass = 1.04 [(IVST + LVEDD + LVPWT)^3 − (LVEDD)^3] − 13.8 g and expressed as LV mass index after adjustment for body surface area (LV mass/BSA). Moreover, LV mass was also corrected for height × 2.7 (LV mass/H$^{2.7}$) according to de Simone *et al.* (19). This index may be more accurate for evaluating LV mass when patients are overweight or may have abnormal body composition.

To assess LV systolic function the following measurements were recorded: the LVEF was calculated using the following formula: LVEF percent = (LVEDV − LVESV)/LVEDV × 100. The FS percentage was calculated using the following formula: FS percent = (LVEDD − LVESD)/LVEDD × 100. Rate-corrected mVCFc was calculated using the following formula: LVEDD − LVESD/LVEDD (LV ejection time), corrected for heart rate. Afterload was measured as ESS and was calculated according to Grossman *et al.* (20). The Ordinary Least Squares simple linear regression was used to estimate ESS-mVCFc equations (21). The relation between FS and ESS, which represents the adequacy of FS in respect to afterload, was quantified as the stress-shortening index (SSI).

Doppler studies provided indices of ventricular filling that were derived from the mitral flow velocity curves, *i.e.* maximal early diastolic flow velocity (E; centimeters per second), maximal late diastolic flow velocity (A; centimeters per second), and the ratio between E and A curves (E/A, normal value >1); the isovolumetric relaxation time (IRT), which represents the interval between the end of aortic valve closure and the onset of mitral valve opening, was also evaluated.

Statistical analysis

All data are reported as means ± SE, unless otherwise reported. Comparisons between patients and controls were performed by paired or unpaired Student's *t* test as appropriate. Pearson's correlation coefficient was calculated to test the relationship between variables. The OLS simple linear regression was used to estimate ESS-mVCFc equations. Test for coincidence and test for parallelism were used to compare ESS-mVCFc equations between patients and controls. Significance was set at 5%.

Results

Cardiovascular parameters in GHD patients and controls at baseline and after 1 yr of GH treatment are reported in Table 2.

At study entry, heart rate, SBP and DBP were similar in the two groups and remained unchanged after 1 and 2 yr of GH.

At baseline, LVEDD and LVPWT were significantly reduced in GHD and resulted in a significant decrease in LV mass, as demonstrated by both LV mass/BSA and LV mass/H$^{2.7}$ that were significantly lower than in controls ($P < 0.002$ and $P < 0.009$, respectively). LVESV and LVEDV were also significantly reduced compared with controls ($P < 0.04$ and $P < 0.002$, respectively) (Table 2).

Global systolic performance was not significantly impaired in our patients. Although FS values showed a trend toward slight impairment when compared with controls, this difference did not reach statistical significance. However, wall stress, as assessed by ESS, was higher than in control subjects ($P < 0.05$) and the SSI was also impaired, indicating that FS, although apparently normal, was inadequate to the afterload. Furthermore, myocardial contractility, measured by mVCFc, was significantly impaired in GHD children ($P = 0.0001$) , and consequently, the ESS-mVCFc relationship was impaired in comparison with healthy controls as shown in Fig. 1 (test for coincidence: F change = 27.342, $P < 0.001$, test for parallelism: F change = 9.236, $P = 0.004$).

After 1 yr of GH, IVST, LVEDD, LVPWT increased, resulting in a significant improvement in LV mass (LV mass/BSA 67.8 ± 2.9 gr/m^2, $P < 0.0001$ and LV mass/H$^{2.7}$

TABLE 2. Cardiac changes over 1 yr of GH therapy in patients with GHD compared with controls

	Baseline	1 yr	P^a	P^b	P^c
Heart rate (bpm)					
GHD	84.2 ± 1.8	83.2 ± 1.3	NS	NS	NS
Controls	81.0 ± 2.8	80.9 ± 3.1			
SBP (mm Hg)					
GHD	93.5 ± 1.7	100.4 ± 2.4	NS	NS	NS
Controls	95.8 ± 1.3	98.8 ± 2.1			
DBP (mm Hg)					
GHD	58.1 ± 1.3	61.5 ± 1.4	NS	NS	NS
Controls	62.3 ± 1.7	59.8 ± 1.7			
IVST (mm)					
GHD	5.3 ± 0.1	6.3 ± 0.2	<0.04	<0.0001	NS
Controls	5.8 ± 0.2	6.1 ± 0.2			
LVEDD (mm)					
GHD	34.9 ± 0.5	40.0 ± 0.8	<0.02	<0.0001	NS
Controls	37.2 ± 0.7	38.3 ± 0.7			
LVESV (ml)					
GHD	15.4 ± 0.9	22.8 ± 1.7	<0.04	0.0004	NS
Controls	19.4 ± 1.6	19.7 ± 1.7			
LVEDV (ml)					
GHD	45.9 ± 2.6	63.9 ± 3.0	<0.002	<0.0001	NS
Controls	58.3 ± 2.5	62.6 ± 3.0			
LVPWT (mm)					
GHD	4.9 ± 0.1	6.1 ± 0.2	0.003	<0.0001	NS
Controls	5.6 ± 0.2	5.9 ± 0.1			
LV mass/BSA (g/m^2)					
GHD	50.6 ± 1.8	67.8 ± 2.9	<0.002	<0.0001	NS
Controls	60.5 ± 2.4	62.1 ± 2.6			
LV mass/$H^{2.7}$ ($g/m^{2.7}$)					
GHD	28.7 ± 1.2	38.2 ± 2.0	<0.009	0.0003	NS
Controls	33.6 ± 1.3	34.2 ± 1.8			
FS (%)					
GHD	34.9 ± 1.5	39.6 ± 1.8	NS	NS (P <0.06)	NS
Controls	37.6 ± 1.1	39.5 ± 1.2			
LVEF (%)					
GHD	64.8 ± 1.2	68.3 ± 1.3	NS	NS (P <0.06)	NS
Controls	67.3 ± 1.4	67.6 ± 1.5			
mVCFc (circ/sec)					
GHD	1.0 ± 0.03	1.2 ± 0.04	0.0001	<0.0002	NS
Controls	1.18 ± 0.03	1.17 ± 0.03			
ESS (g/cm^2)					
GHD	49.2 ± 1.4	43.9 ± 1.4	<0.05	<0.03	NS
Controls	45.7 ± 1.0	45.5 ± 0.9			
SSI					
GHD	0.10 ± 0.02	0.19 ± 0.02	<0.007	<0.003	NS
Controls	0.18 ± 0.02	0.18 ± 0.02			
E/A					
GHD	1.8 ± 0.06	1.8 ± 0.05	NS	NS	NS
Controls	2.0 ± 0.09	2.0 ± 0.09			
IRT (msec)					
GHD	58.2 ± 2.2	57.3 ± 2.2	NS	NS	NS
Controls	62.4 ± 1.8	59.3 ± 1.4			

NS, Not significant.

[a] GHD patients at baseline *vs.* controls.

[b] GHD patients at baseline *vs.* 1 yr of GH therapy.

[c] GHD patients after 1 yr of GH treatment *vs.* controls.

38.2 ± 2.0 $g/m^{2.7}$, $P = 0.0003$) (Table 2). LVESV (22.8 ± 1.7 ml, $P = 0.0004$) and LVEDV (63.9 ± 3.0 ml, $P < 0.0001$) also significantly increased after treatment. Compared with the control group, all these morphological parameters fully normalized after 1 yr of therapy (Table 2).

The increase of LV mass was significantly correlated with the increase of IGF-I levels (LV mass/BSA: r = 0.51, $P = 0.0002$; LV mass/$H^{2.7}$: r = 0.32, $P = 0.04$).

One year of GH treatment was associated with improved ventricular contractile state and reduced afterload.

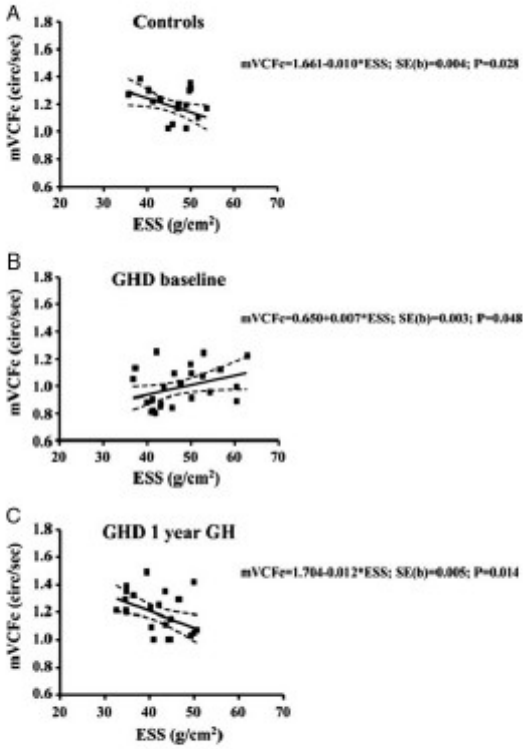

FIG. 1. Linear regression equations for the relationship between ESS and rate-corrected mVCFc in controls (A) and GHD patients at baseline (B) and after 1 yr of GH therapy (C). The *dashed curves* represent 95% confidence bounds for the least-squares fit. *P* values for statistical significance of the slope coefficients in each linear regression model are given in the corresponding graphs. Regression equations are significantly different when GHD patients at baseline (B) are compared with controls (A) as documented by test for coincidence: F change = 27.342, $P < 0.001$, and test for parallelism: F change 9.236, $P = 0.004$. Regression equations were no longer different when GHD patients after 1 yr of treatment (C) were compared with controls (A) as documented by test for coincidence: F change = 0.805; $P = 0.452$.

After treatment, mVCFc and the SSI significantly increased and ESS significantly decreased compared with pretreatment values ($P < 0.0002$, $P < 0.003$, and $P < 0.03$, respectively), becoming similar to controls (Table 2). The ESS-mVCFc relationship (Fig. 1) also significantly improved compared with pretreatment values, becoming coincident to that of controls (test for coincidence: F change = 0.805; $P = 0.452$). Moreover, FS and LVEF also showed a trend toward improvement after GH, although it was not statistically relevant ($P < 0.06$) (Table 2).

The increase of mVCFc and SSI were significantly correlated with the increase of LV mass (LV mass/BSA, $r = 0.43$, $P = 0.002$, and $r = 0.46$; $P = 0.0009$, respectively; LV mass/$H^{2.7}$, $r = 0.52$, $P = 0.0005$, and $r = 0.38$; $P = 0.01$) and IGF-I ($r = 0.39$, $P < 0.006$, and $r = 0.46$, $P = 0.001$, respectively). As expected, the improvement of SSI was also significantly correlated with the increase of mVCFc ($r = 0.77$, $P < 0.0001$) and FS ($r = 0.75$, $P < 0.0001$). The reduction of ESS was a consequence of the positive effects of GH on heart size too, being correlated with the increase of LVEDD ($r = -0.3$, $P < 0.05$), LVPWT ($r = -0.65$, $P < 0.0001$), and IGF-I ($r = -0.3$, $P < 0.05$).

The diastolic function, measured as E to A ratio and IRT, was similar in GHD children and controls at baseline and did not change during treatment (Table 2).

No further changes in cardiac morphology or systolic function were observed during the second year of therapy (Fig. 2).

In 11 children with severe GHD, LV mass/BSA (46.6 ± 1.5 g/m^2), and LV mass/$H^{2.7}$ (26.8 ± 0.8 g/m$^{2.7}$) were significantly reduced at baseline compared with both children with partial GHD ($P = 0.007$ and $P < 0.04$, respectively) and controls ($P < 0.0001$ and $P = 0.0002$, respectively); mVCFc (0.95 ± 0.03 circ/sec) and SSI (0.08 ± 0.02) were significantly reduced, and ESS (50.8 ± 2.1 g/cm^2) was significantly increased only in comparison with controls ($P < 0.0001$, $P < 0.003$, $P < 0.05$, respectively) (Fig. 3). When compared with controls, children with partial GHD also showed significantly reduced LV mass/BSA (54.0 ± 1.9 g/m^2, $P < 0.05$) and LV mass/$H^{2.7}$ (30.0 ± 1.1 g/m$^{2.7}$, $P < 0.05$). With regard to systolic function, children with partial GHD showed lower mVCFc (1.04 ± 0.04 circ/sec; $P < 0.01$) and SSI (0.11 ± 0.02, $P < 0.03$) compared with healthy controls, whereas ESS (48.0 ± 2.8 g/cm^2) was not significantly different (Fig. 3). Similar results regarding both morphological and functional data were also obtained when the severity of GHD was evaluated on the basis of IGF-I levels.

After 1 yr of GH, LV cardiac size and myocardial contractility significantly improved becoming similar to that of controls in patients with both severe and partial GHD. No further significant changes were observed during the second year of therapy (Fig. 3).

Cardiac diastolic function before and during GH therapy was similar in children with severe or partial GHD (data not shown).

No significant differences were observed in cardiac morphology and function between males and females with GHD at baseline (LV mass/BSA, 52.2 ± 2.3 *vs.* 49.0 ± 2.9 g/m^2; LV mass/$H^{2.7}$, 30 ± 1.1 *vs.* 29 ± 1.4 g/m$^{2.7}$; FS percent, 34.7 ± 0.9 *vs.* 35.0 ± 1.1; mVCFc, 1.03 ± 0.04 *vs.* 0.97 ± 0.05 circ/sec; ESS, 49.4 ± 1.4 *vs.* 49 ± 1.1 g/cm^2; SSI, 0.10 ± 0.02 *vs.* 0.09 ± 0.02; E/A 1.7 ± 0.10 *vs.* 1.8 ± 0.07; IRT, 61.8 ± 2.1 *vs.* 54.9 ± 2.1 msec, respectively). In both males and females, LV mass/BSA, LV mass/$H^{2.7}$, mVCFc, and SSI significantly improved and ESS significantly decreased after 1 yr of GH therapy and then remained stable during the second year of GH.

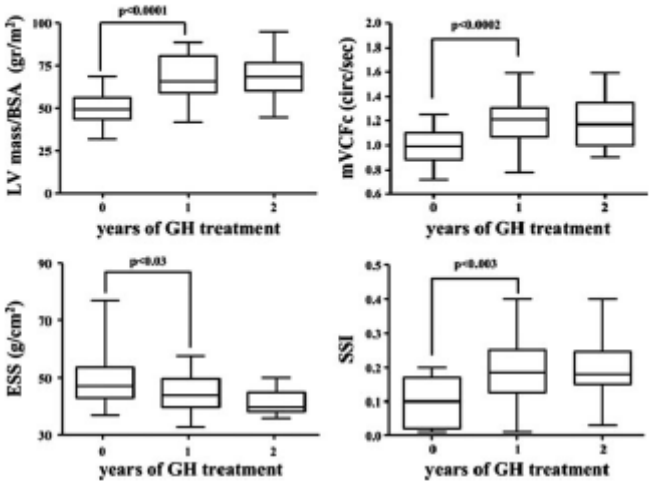

FIG. 2. Left ventricular mass/BSA (*top left*), ESS (*bottom left*), rate-corrected mVCFc (*top right*), SSI (*bottom right*), in children with GHD before and after 1 and 2 yr of GH therapy. The *boxes* show medians, 25th and 75th percentiles, and the whiskers represent the highest and lowest values.

Concerning the evaluation of parameters of atherosclerotic or thrombotic risk, fasting triglycerides (67.1 ± 6.5 mg/dl), total (163 ± 4.0 mg/dl), LDL cholesterol (91 ± 4.0 mg/dl), and AI (3.0 ± 0.1) were similar in GHD patients at baseline and in controls (59.3 ± 3.9, 166 ± 3.0, 98.6 ± 3.2, and 3.0 ± 0.08 mg/dl, respectively). After 2 yr of GH, a beneficial effect was observed with a decrease in total (147 ± 4.5 mg/dl, $P < 0.04$), LDL cholesterol (75 ± 3.7 mg/dl, $P = 0.02$), and AI (2.6 ± 0.1, $P < 0.04$). Homocysteine was significantly higher in GHD patients before GH therapy (8.1 ± 0.5 μmol/liter) than in controls (5.4 ± 0.3 μmol/liter, $P < 0.0001$) and significantly decreased during treatment (6.6 ± 0.5, $P < 0.05$).

Discussion

The results of the current prospective case-control study demonstrate that children with GH deficiency may have subtle alterations in LV systolic performance in addition to reduced cardiac size. One year of GH therapy normalizes cardiac mass and reverses the abnormalities of myocardial contractility. No further changes occur during the second year of therapy. These results confirm our previous findings that GHD children have impaired LV mass (8). Because normalization to BSA may be less appropriate in GHD children given their abnormal body composition, with a reduction in lean mass and an increase in body fat, we evaluated LV mass also corrected for height × 2.7 to better normalize data. Our data concerning both LV mass/BSA and LV mass/H $^{2.7}$ confirmed that GHD children may have reduced cardiac mass. Our findings are in agreement with results reported by Shulman *et al.* (5) in a prospective, but uncontrolled, study. Cardiac size and performance were evaluated in 10 children with GHD at baseline and after 1 yr of GH therapy. The evaluation of LV mass indexed by height, height × 2.7, and BSA in GHD children produced similar results; in fact, they were all reduced compared with reference values at baseline and significantly improved after 1 yr of GH. Overall these data suggest that the reduction in cardiac size is not a result of any biases in the measurement of the LV mass but a result of being GH deficient.

With regard to cardiac function, previous studies in children with GHD failed to reveal any alteration in cardiac function (5–8). In adults with GHD, echocardiographic studies of systolic function have yielded contrasting results on the effect of both GHD and GH therapy on cardiac performance (1, 12). However, using radionuclide angiography, a remarkable impairment of LV performance was found in most adults with GHD, regardless of their age and the age of disease onset (22), instead cardiac performance is correlated with the GH status (11). These abnormalities were reversed at least in part by GH therapy (22).

One of the major issues in evaluating cardiac function with echocardiography is that FS and LVEF, the most frequently used indices of systolic function, may not be sensitive enough to reveal minimal changes in cardiac performance. Although they may be very useful in a clinical setting, FS and LVEF may overestimate cardiac function because they are both highly dependent on myocardial loading.

To our knowledge, this is the first study that documents an impairment in cardiac performance in GHD children. In our group of patients, the evaluation of mVCFc, ESS, SSI, and the ESS-mVCFc relationship revealed a mild impairment in myocardial contractility. Higher wall stress, associated with an impaired mVCFc, caused a significant alteration in the ESS-mVCFc relationship, a sensitive measure of contractile state that is preload independent, normalized for heart rate and incorporates afterload, accurately reflecting the state of myocardial contractility irrespective of loading conditions (21). These abnormalities were of little clinical significance because FS and LVEF were not significantly impaired, documenting a normal global systolic function. However, FS resulted as being inadequate to the increased wall stress, causing a significant impairment in the SSI. Furthermore, al-

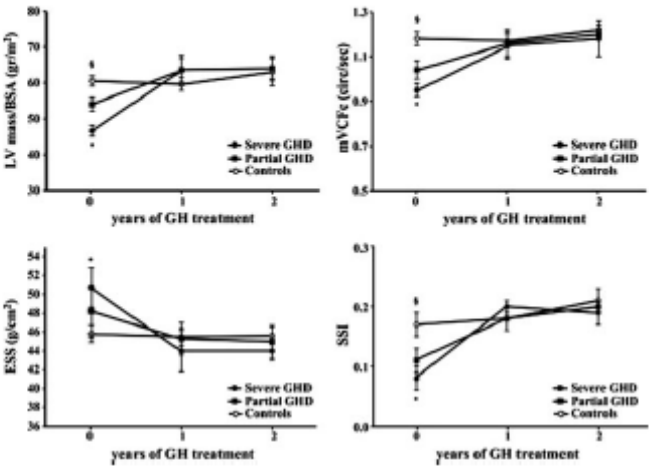

FIG. 3. LV mass/BSA (*top left*), ESS (*bottom left*), rate-corrected mVCFc (*top right*), and SSI (*bottom right*) (means ± SE), before and during 2 yr of GH therapy, in children with severe GHD (GH peak <5 μg/liter after stimulation tests) compared with children with partial GHD (GH peak above 5 μg/liter after stimulation tests) and controls: LV mass/BSA: *, $P < 0.0001$ comparing children with severe GHD *vs.* controls and $P = 0.007$ *vs.* partial GHD; §, $P < 0.05$ comparing children with partial GHD *vs.* controls greater than mVCFc: *, $P < 0.0001$ comparing children with severe GHD *vs.* controls; §, $P < 0.01$ comparing children with partial GHD *vs.* controls; ESS: *, $P < 0.05$ comparing children with severe GHD *vs.* controls; SSI: *, $P < 0.003$ comparing children with severe GHD *vs.* controls; §, $P < 0.03$ comparing children with partial GHD *vs.* controls.

though they were not clearly reduced, FS and LVEF showed a trend toward impairment compared with controls.

In addition to a positive effect on cardiac size, 1 yr of GH caused a significant decrease in wall stress and a consistent increase in mVCFc, resulting in a significant improvement in the ESS-mVCFc relationship that became similar to controls. FS and LVEF showed a slight, but not statistically relevant, increase during GH therapy, too. The increase in FS, associated with the reduction of ESS, caused a significant improvement in SSI. Changes in mVCFc, ESS, and SSI were all significantly correlated with IGF-I and changes in LV dimensions, suggesting that changes in myocardial contractility and wall stress were mediated by the effects of GH and IGF-I on wall thickness and heart size.

No further changes in systolic function were observed during the second year of therapy. Neither GHD nor GH therapy was associated with significant alterations in diastolic function.

A similar evaluation of cardiac performance through load-independent indices of ventricular function was also performed by Shulman *et al.* (5). Although no significant abnormalities of LV systolic function were identified before or during GH therapy, a trend toward improvement in mVCFc was observed after therapy compared with baseline values, in agreement with our data.

The effects of GH treatment on preload- and postload-independent indices of cardiac performance were also evaluated by Radetti *et al.* (23). In agreement with our findings, LV mass and systolic and diastolic functions in 14 GHD adolescents did not differ from a control group after 1 yr of GH therapy except for the SSI, which was slightly reduced. However, the major drawback of the study by Radetti *et al.* was the lack of baseline data.

Our findings are also in agreement with studies of adults with GHD. In fact, Fazio *et al.* (24) showed a consistent decrease in mVCFc and a significant increase of ESS before GH treatment compared with controls; as in our study, GH therapy resulted in a significant improvement of both parameters. In contrast to our results, these patients also showed a significant impairment in LVEF. This difference in LVEF between children and adults may reflect the shorter exposure of children to the effects of GH deficiency. This hypothesis could be supported by the trend toward FS and LVEF impairment before GH treatment and improvement after GH therapy in our children. Moreover, in adolescents with severe GHD 6 months after the withdrawal of GH, LVEF showed a trend toward an impairment before GH therapy and an improvement after GH restart (25). In contrast, a significant decrease in FS and mVCFc in addition to impaired LV mass have also been reported in adults with childhood-onset GHD after GH discontinuation of at least 5 yr (26). Taken together, all these findings suggest that a prolonged GH deprivation can be associated with a greater impairment of systolic function.

Concerning the evaluation of atherosclerotic or thrombotic risk factors, GHD in our patients was associated with higher serum homocysteine levels compared with controls, without significant effects on lipid profile as already reported (8, 27). Two years of GH treatment significantly decreased homocysteine concentrations and improved lipid profile with a decrease of total cholesterol and the total to HDL cholesterol ratio, compared with both pretreatment and control values. These results suggest that children with untreated GHD may also have subtle abnormalities of lipid profile that might place them to an increased atherosclerotic or thrombotic risk and on which GH exerts a beneficial effect.

Another end point of our study was to investigate whether the severity of GHD was also correlated with the degree of cardiac impairment. We previously reported that

children with severe GHD have a more evident reduction in LV mass/BSA than children with partial GHD, although LV mass/BSA was significantly reduced compared with controls in both groups (8). The results of the current study show that children with severe GHD have a greater impairment of systolic function in addition to a more severe reduction of LV mass when compared with children with partial GHD. However, children with partial GHD also have a significant reduction in cardiac size compared with controls, whereas myocardial contractility was only slightly impaired and afterload was not significantly increased. GH therapy normalizes cardiac size and function in both severe and partial GHD patients.

As was the case in other studies (8, 28), we did not observe gender-related effects of both GHD and GH treatment on cardiac size and performance.

In conclusion the results of the present study demonstrate for the first time that children with GHD have mild alterations in myocardial contractility. However, these abnormalities are likely of little clinical significance in this age group because global systolic function is normal. GH therapy is able to reverse all these subtle abnormalities.

Taken together these results indicate that GH deficiency is associated to abnormalities in both cardiac morphology and function in not only adults but also children and further support the beneficial effect of GH on the heart.

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CONCLUSIVE REMARKS

Adults with GH deficiency present a cluster of risk factors that may place them to a higher rate of morbidity for cardiovascular events. Although recent evidence suggest that atherosclerosis begin in childhood, only a few studies have investigated cardiovascular risk in children affected by GHD. However, there is preliminary evidence that GHD also in children and adolescence may be associated with cardiovascular and metabolic abnormalities.

Our data on children affected with GHD, confirmed that this condition is associated also in childhood with a mild cardiovascular alteration. In particular, we found that children with GHD have impaired cardiac mass, compared with healthy controls. Moreover, for the first time, we documented a subtle alteration of systolic cardiac function in GHD children, although cardiac global function is not altered. Replacement therapy with growth hormone exerts a beneficial effects on these risk factors. In fact GH is able to normalize both cardiac size and performance. Further studies are needed to clarify how these subtle abnormalities predispose to increased risk of cardiac disease in adulthood.

CHAPTER 7

NOVEL PATHOGENETIC MECHANISMS OF ENDOCRINE DISEASES: NEW INSIGHTS IN GENETICS OF HYPERTHYROIDISM AND GH DEFICIENCY

7.1 Introduction and aims

Genetic forms of GH deficiency

Short stature associated with GH deficiency (GHD) has been estimated to occur in about 1 in 4,000 to 1 in 10,000 in vatrious studies (1-3). Although most cases are sporadic and are believed to result from environmental cerebral insult or developmental abnormalies, 3% to 30% of cases have an affected first-degree relative suggesting a genetic etiology. Because MRI examination identify only about 12% to 20% anomalies of either hypothalamus or pituitary gland in isolated growth hormone deficiency (IGHD), it can be higher proportion of sporadic cases may have indeed a genetic cause (4). Familial IGHD, hawever, is associated with at least four mendelian disorders (5).

With regard to combined pituitary hormone deficiency, it can be associated with alterations in various transcription factors of the pituitary gland. At the very beginning, the GH deficiency (GHD) might be the only hormonal deficiency and these factors should be take into account when examining and following-up these patients. In this context the two most important transcription factors to be described are PROP 1 and POU1F1.

PROP-1

In mice, Prop1 gene mutation primarily causes GH, PRL and TSH deficiency and in humans PROP 1 gene mutations also seem to be a major cause of combined pituitary hormone deficiency. In agreement with the model of Prop1 playing a role in commitment of dorsal lineages (GH, PRL, and TSH), Prop1 mutant mice exhibit a dorsal expansion of gonadotrophs that normally arise on the ventral.

To date, many different missense, frameshift and splice site mutations heve been reported, The clinical phenotypes varies even between siblings with the same genetic mutation (6). The affected patients are not only GH, Prolactin (PRL) and Thyroid-stimulating hormone (TSH) deficient but also gonadotropin deficient.

The three tandem repeats of the dinucleotides GA at location 296-302 in the Prop1 gene represent a hot spot for combined pituitary hormone deficiency (6-8).

POUF1 (PIT1)

The pituitary transcription factor PIT-1 is a member of the POU-family of homoproteins, which regulates important steps during embryologic development of the pituitary gland and regulates target gene function during postnatal life. Because PIT1 is confined to the nuclei of somatotropes, thyrotrops and lactotrops in the anterior pituitary gland, the target genes of PIT1 include the GH, PRL and TSH. The defects in the human POU1F1 result in a total deficiency of GH and PRL, whereas a variable hypothyroidism caused by insufficient TSH secretion, at least during childhood, has been described. Although it is important to stress that the clinical variability is caused by other factors than the exact location of the mutation reported, the type of inheritance, however, seems to correlate well with the genotype (9). The first mutation within the POU1F1 was identified by Tastumi (10). Most of the mutations reported so far are recessive: however, a number of heterozygous point mutations have been reported (11).

Of those the amino acid substitution R271W seems to be a hot spot. Further, the dominant-negative effect of the R271W POUF1 form has been recently challenged by Kishimoto and co-workers (12).

Although most cases with R271W are sporadic and present with an autosomal-dominant mode of inheritance, Okamoto and co-workers (13) reported a family with normal family members who were clearely heterozygous for thet mutation. Further in vitro expression studies were performed, which could not confirm its dominant negative effect that is well in contrast with the original report (5, 12).

Genetic forms of Hyperthyroidism

Familial Nonautoimmune Hyperthyroidism (FNAH) or hereditary toxic thyroid hyperplasia is a clinical entity originally described by Thomas et al (14). This condition is clinically characterized by thyroid autonomy in two or more generations with a variable age of onset (from infancy to adulthood) as well as frequent relapses of hyperthyroidism after thyrostatic therapy withdrawal or partial thyrodectomy. Thyroid-stimulating hormone (thyrotropin) receptor (TSHR) antibodies are always absent (15). This is a rare disorder with an

incidence of less than 1% in patients with juvenile hyperthyroidism, and with a frequency of 6% in patients with thyrotoxicosis without thyroid antibodies (16). Since the condition is dominantly autosomal inherited, molecular diagnostics and genetic counselling are advocated in the affected families.

Aim of this phase of the project was to describe new genetic mutations underlying GH deficiency and hyperthyroidism, respectively. In particular we described a new mutation in POUF1 causing multiple GHD and a new mutation in the TSHR causing familial nonautoimmune hyperthyroidism.

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7.2 A Novel recessive splicing mutation in the POU1F1 Gene causing Combined Pituitary Hormone Deficiency

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A novel recessive splicing mutation in the *POU1F1* Gene causing Combined Pituitary Hormone Deficiency

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ABSTRACT

Background: Mutations in the gene encoding the pituitary transcription factor POU1F1 have been described in Combined Pituitary Hormone Deficiency (CPHD).

Aim: The aim of this study was the characterisation of the molecular defect causing CPHD in a patient born to consanguineous parents.

Subject and Methods: A 12.5 years old girl presenting with severe growth failure at diagnosis (-3SDS at three months) and deficiency of GH, PRL and TSH was investigated for the presence of *POU1F1* gene mutations by DHPLC analysis

Results: A novel mutation adjacent to the IVS2 splicing acceptor site (IVS2-3insA) was identified in the patient at the homozygous state. Analysis of patient's lymphocyte mRNA and an in-vitro splicing assay revealed the presence of two aberrant splicing products: i) deletion of the first 71 nucleotides of exon 3, altering the open reading frame and generating a premature stop codon, ii) total exon 3 skipping resulting in an in frame deleted mRNA encoding a putative protein lacking part of the transactivation domain and of the POU-specific homeodomain.

Notably the patient's relatives heterozygous for the mutation had PRL levels under the normal range with no evident clinical symptoms.

Conclusions: The IVS2-3insA mutation, responsible for CPHD at the homozygous state, causes the presence of two aberrant splicing products encoding non-functional products. In the heterozygotes one normal allele might not guarantee a complete pituitary function.

INTRODUCTION

The pituitary-specific factor 1 (POU1F1; also known as PIT-1) is a nuclear transcription factor of 291 amino acids, responsible for pituitary development in mammals. It is expressed in the anterior pituitary lobe where it contributes to the differentiation and proliferation of somatotrophs, lactotrophs, and thyrotrophs [1].

As a member of the POU domain transcription factors, POU1F1 contains a DNA binding domain consisting of a 3α-helix POU-homeodomain (POU-HD) and of a 4α-helix POU-specific (POU-S) domain essential for high-specificity DNA binding and protein/protein interaction [2; Fig 1a]. The third α-helix of both the POU-HD and POU-S domains is responsible for the majority of contacts with the major DNA groove. The N-terminal part of POU1F1 is involved in the transcriptional activation of several pituitary expressed genes. POU1F1 activates the growth hormone (GH1), the prolactin (PRL), the thyroid stimulating hormone (β-TSH), the growth hormone releasing hormone receptor (GHRHr) genes and the POU1F1 gene itself by binding as a homodimer to their promoter response elements and recruiting coactivator proteins to the transcriptional complex [4-7]. Utilizing the multiple POU1F1 binding sites on the PRL promoter it has been shown that POU1F1 is responsible for the remodelling of the chromatin structure by nucleosome repositioning [8]. A coactivator complex that includes the CREB (cAMP response element binding protein) binding protein (CBP) and a co-repressor complex that includes the nuclear receptor corepressor (N-CoR) [9] with a deacetylase activity interacts with the PRL promoter. The changes in the chromatin structure are the result of a regulated balance between the interaction of these two complexes [9]. Because of its central role in transcription regulation, patients harbouring deleterious mutations in the *POU1F1* gene present with combined deficiency of the pituitary hormones GH, PRL and TSH, variable hypoplasia of the anterior pituitary and severe growth retardation. At least 25 different

mutations (5 dominant and 20 recessive) have been so far identified [10-28] in combined pituitary hormone deficiency (CPHD). In most cases the inheritance pattern reflects the localization of the mutations: those lying within the POU domains, which are the majority and have in several cases been found to affect the ability of POU1F1 to dimerize and bind to DNA, tend to be inherited as autosomal-recessive, while mutations detected outside the DNA-binding domains (P24L, R143Q, K216E and R271W) follow an autosomal-dominant inheritance with few exceptions.

Here we report a novel *POU1F1* recessive mutation detected in an Italian patient with profound GH, PRL and TSH deficiency, born to consanguineous parents. Its location nearby a splicing consensus site was indicative of an effect on mRNA processing. The transcript analysis showed that the mutation leads to the presence of two aberrant mRNA isoforms. Hormonal evaluation showed that the patient's healthy heterozygous relatives had a partial PRL deficiency.

MATERIAL AND METHODS

Clinical studies and hormonal assays

GH was evaluated after two stimulation tests with arginine (0.5g/kg, i.v.; Arginine hydrochloride, Salf, Bergamo, Italy) and glucagon (0.05 mg/kg, i.m.; GlucaGen, Novo Nordisk, Denmark). Serum growth hormone (GH) levels were measured by immunoradiometric assay (IRMA) using a commercially available kit (HGH-CTK-IRMA Sorin, Saluggia, Italy).

Serum insulin-like growth factor-I (IGF-I) levels were measured using a two-site IRMA kit (Diagnostics System Laboratories, Inc. Webster, TX). The IGF-I intra and inter assay CVs were 3.4% and 8.2% respectively. The normal range was: 60-350 µg/L in children aged below 5yr, 180-780 µg/L in adolescents aged 11- 18 yrs, and 90-280 µg/L in adults.

The serum prolactin (PRL) level was assessed by immunometric assays using a commercial kit (Immulite 2000, Simiens Diagnostic Product Corporation, Los Angeles, CA, USA). The normal range was 5-25 ng/ml for females and 5-15 ng/ml for males.

The serum TSH level was measured using IRMA (Delfia Inc. Wallac, Finland). Serum FreeT3 (FT3) and freeT4 (FT4) concentrations were determined by the RIA Lisophase kit (Technogenetics, Milan, Italy). The normal ranges were: TSH 0.3-4.2 mU/l; FT3 2.0-4.4 pg/ml and FT4 0.9-1.7 ng/dl. Plasma ACTH and serum cortisol concentrations were measured by immunometric assays using commercial kits (Immulite 2000, Siemens Diagnostic Product Corporation, Los Angeles, CA, USA). The normal ranges at 8:00 a.m. were 10-130 pg/ml and 5-20 µg/dl respectively.

Height and parental target height (sex-corrected mid-parental height) were expressed in both centimetres and standard deviation score (SDS) according to the Tanner standards [29].

Genetic analysis of the POU1F1 gene

Blood samples were collected after a written informed consent by the parents for themselves and for their three children. Genomic DNA was isolated from peripheral blood leukocytes according to standard methods. The DNA fragments covering the six exons and the intron-exon boundaries of the *POU1F1* gene were amplified. PCR conditions and primer sequences are available upon request.

Mutation analysis was performed by DHPLC using the Transgenomic WAVE system (Transgenomic, San Jose, CA, USA). PCR products were denatured at 95°C, re-annealed at 65°C for 1 min and cooled at 4°C to generate heteroduplexes. In order not to miss a mutation at the homozygous state, as the patient was born to consanguineous parents, all the amplicons were mixed with a corresponding reference PCR product from a sequenced normal control prior to the heteroduplex generation cycle [30]. The optimal column temperature for fragment analysis was calculated using the WaveMaker Software (Transgenomic, San Jose, CA, USA). The detected mutation was then characterized by directly sequencing the purified PCR products in both directions (Big dye terminator cycle sequencing kit, Applied Byosistem, Foster City, CA) on an ABI310 Automated Sequencer and analysis with the Sequencer software.

Lymphocyte mRNA analysis

Total RNA was extracted from lymphocytes by RNAwiz (Ambion, Austin, TX, USA). First strand cDNA synthesis was performed using the RETROscript Kit (Ambion) following the manufacturer's instructions in a total volume of 20 µl with a specific antisense primer POU1F1-6 (5'-TCTGCACTCAAGATGTTCCTT -3') located in exon 6. The *POU1F1* cDNA was first amplified with a forward primer located in exon2 (POU1F1-2 5'-GATGTCTACAGCAACAGGACT-3') and the POU1F1-6 primer in exon 6 as the reverse, with one µl of the retrotranscription reaction under the following conditions: 94 °C for 5 minutes followed by 38 cycles at 24 °C for 1 minute, 56.5 for 1 minute and 72°C for 1 minute followed by 72°C for 5 minutes. To clearly distinguish the product on the gel, a nested PCR was then performed with one µl of the first PCR reaction and primers located within exon 2 and exon 4 (forward: 5'-TTATTCTGTTCCTTCCTGTC-3' and reverse 5'-GCTGCAGATTTTCAAATCGGC-3') using the same reaction conditions. The products were visualized on a 2% agarose gel. The different bands from all the analysed subjects were excised from the gel, purified by the Qiaquick gel extraction kit (Qiagen, Hilden, Germany) and directly sequenced on an ABI310 Automated Sequencer.

Evaluation of the splicing mutation by use of an exon-trapping system

The exon trapping technique was performed to analyse the in vitro effect of the IVS2-3insA mutation. Briefly, a genomic region (GeneBank accession number NT_022459) encompassing *POU1F1* exon 3 and including 262 bp of intron 2 upstream the IVS2 acceptor splice site and 242 bp of intron 3 downstrem the IVS3 donor splice site was amplified with primers (forward: 5'-GAATTCTATTTGCAGTCCCCTGCTTC-3' and reverse 5'-GGATCCAATGTTGAGGTAAGTTTTGAAAGAG-3') from the genomic DNA of the patient homozygous for the IVS2-3insA mutation and of a control, using a proofreading DNA polymerase

(Finnzyme, Espoo, Finland). A non template restriction endonuclease recognition sequence was added to the 5' end of both primers (underlined above): EcoRI (forward) and BamHI (reverse). After cloning in pMOSBlue T-vector, PCR products corresponding to the normal and the mutant alleles were sequenced to check for polymerase induced errors and digested with EcoRI and BamHI restriction enzymes. The inserts were separately cloned into the pSPL3 eukaryotic vector (kind gift of Dr. Isabella Ceccherini) and digested with the same endonucleases. The resulting two expression constructs were transiently transfected by means of Fugene 6 (Roche Diagostics, Basel, CH) into COS-7 cells, following the manufacturer's instructions. Total RNA was isolated 48 h after transfection using RNAwiz (Ambion, Austin, TX, USA). RT-PCR on total RNA was performed with specific pSPL3 primers, SD6 (5'-TCTGAGTCACCTGGACAACC- 3') and SA2 (5'-ATCTCAGTGGTATTTGTGAGC-3'), under the following conditions: 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute for 30 cycles, followed by 72°C for 5 minutes. The amplified products were separated on a 2% agarose gel, excised from the gel and sequenced (ABI Prism 3100).

RESULTS

Clinical and hormonal features

The proband is the second of three daughters of healthy consanguineous Italian parents (second-degree cousins; Fig 2). She was born at term after an uncomplicated pregnancy and delivery by caesarean section. Birth weight was 2900 grams and birth length was 49 cm. Postnatal course was complicated by prolonged jaundice and failure to thrive. She was admitted to the Department of Pediatrics of the University of Naples at the age of 3 months for severe growth failure and feeding problems with a weight of 4180 g, length of 52.5 cm (-3 SDS) and head circumference of 37.5 cm. She showed typical signs of hypopituitarism such as doll-like face, broad and flat nose, widely open anterior and posterior fontanels.

Pituitary hormone assessment revealed low FT4 (0.5 ng/dl) and low TSH (1.2 µU/ml) levels suggesting the diagnosis of central hypothyroidism. PRL levels were undetectable (<0.1 ng/ml). Cortisol and ACTH concentrations were in the normal range. L-thyroxine replacement therapy was successfully started and two months later, when euthyroidism was achieved, GH secretion was evaluated. GH deficiency (GHD) was diagnosed by an extremely low peak of GH concentration (< 0.1 µg/L) after two independent stimulation tests. Magnetic resonance imaging (MRI) of the brain revealed hypoplasia of the anterior pituitary gland with normal signal and location of the posterior pituitary gland.

At the age of 5 months GH replacement therapy was started with a significant improvement in linear growth. At the age of 30 months a complete normalization of her stature (height 90.5 cm, +0.45 SDS) was observed.

Puberty begun spontaneously at the age of 9.5 years and menarche occurred at 10.7 years.

She is now 12.5 years old, her height is 161.3 cm (+1.28 SDS), quite above her target height (157 cm, -0.8 SDS), her weight is 53 kg. Menses are regular. She has a high-normal intellectual quotient evaluated by WISC-R (Global IQ 126, Verbal IQ 123, Performance IQ 124). She is still undergoing GH and L-thyroxine therapy. The ACTH level always remained within the low-normal range throughout the follow-up (10-14 pg/ml) and cortisol concentrations were always normal (8-16 µg/dl) measured at 8.00 a.m. A low dose ACTH stimulation test (at 12.5 years) revealed a normal cortisol response to the stimuli (peak cortisol 20 µg/dl).

The height of both parents is normal: the patient's mother is 157 cm (-0.8 SDS) and her father is 170 cm (-0.7 SDS). Results of their hormonal assessment are reported in Table 1. All the hormone levels were within the normal range with the notable exception of PRL that was below the normal range. The patient's mother was able to breastfeed her first two children for 3 months, and the third child only for few days. The patient's heterozygous sister (II-1, Fig. 2) also showed low PRL levels (Table 1). She is a 15.7 year old healthy adolescent of normal stature (height 159 cm; -0.5 SDS). Menarche occurred at the age of 12 years and her menses are regular.

The proband's sister (II-3, Fig 1), homozygous for the wild-type allele is 10.5 year old. Her height is normal (-0.94 SDS) and she is spontaneously beginning the pubertal development (breast and pubic hair Tanner stage 2). Hormonal assessment did not reveal any abnormality (Table 1).

Identification of a novel splicing mutation

The entire *POU1F1* coding sequence and intron-exon boundaries of the patient were screened by DHPLC heteroduplex analysis. The amplicon corresponding to exon 3 showed an heteroduplex chromatogram after being mixed, denatured and renatured with a reference sample suggesting the presence of a homozygous variation. Sequencing revealed an insertion of an adenine at the third base upstream the intron2/exon3 junction (IVS2-3insA), leading to the presence of an A at position -3 of the acceptor splice site instead of a C and changing the IVS2/EXON 3 junction from cacag/GT to cacaag/GT. The same mutation was detected in both parents (I-1 and I-2, fig2) and in the eldest sister (II-1) at the heterozygous state, while II-3 was homozygous for the wild-type allele.

Functional analysis of the IVS2-3insA mutation

The location of the IVS2-3insA variation immediately upstream the highly conserved dinucleotide AG at the acceptor splicing site suggested that it might interfere with the correct mRNA maturation. Investigation of the strength of the intron 2 acceptor site using a splice site prediction program (http://www.frsuitfly.org/seq_tools/splice.html) showed that it was a strong splice site with a score of 0.97 (max = 1) and that the IVS2-3insA mutation changed this score to 0.05 (threshold = 0.40). We thus attempted to confirm the functional significance of this change by analysing the lymphocyte ectopic transcripts in the patient and in her relatives. The *POU1F1* cDNA amplified with primers located in exon 2 (cDNA position 156-175) and exon 4 (cDNA position 512-532) yielded a wild-type fragment of 377 bp in the normal homozygous sister (II-3; fig 3a, lane 6) and in normal controls (data not shown), as revealed by sequencing of the band extracted from agarose. In the affected homozygous subject (II-2, fig. 3a, lane 5) two lower molecular weight products were

detected. Sequence analysis revealed that they were the consequence of two aberrant splicing events: i) a band of 306 bp resulting from the skipping of the first 71 bp of exon 3 consequent to the activation of a cryptic splice site located within exon 3 and ii) a band of 152 bp as a consequence of the total skipping of exon 3 (fig 3a). The IVS2-3insA mutation thus inactivates the 3′ splice acceptor site of intron 2, at least in lymphocytes, as predicted from the software analysis. The deletion of the 71 bp corresponding to cDNA position 215–285, alters the open reading frame of the mutant transcript and generates a premature termination codon within exon 3, at residue 82 (fig1b). The total skipping of exon 3 results in an in-frame deleted mRNA that could potentially be translated into an internally deleted POU1F1 molecule (fig1c). In the three heterozygous subjects (I-1, I-2 and II-1) the wild-type and the two aberrant splicing products were detected (fig 3a, lanes 2, 3 and 4 respectively). This assay is not quantitative as it only allows to detect the presence or the absence of the *POU1F1* mRNA isoforms. The different band intensity seen on the agarose gel is likely an artefact due to the high number of amplification cycles in the two PCR rounds performed to detect the lymphocyte "illegitimate" transcription of *POU1F1*, a gene normally expressed in the pituitary, that could lead to the random preferential amplification of a fragment rather than another.

To assess whether the IVS2-3insA mutation might generate other isoforms besides the ectopic transcripts detected in the patient's lymphocytes, we analysed the mutated allele mRNA products by an in vitro exon-trapping system. COS cells were transfected with constructs carrying the exon 3 of *POU1F1* with parts of the two flanking introns amplified either from the wild type or from the mutant allele (Fig. 3b). The mRNAs from the transfected cells were reverse transcribed and amplified with the plasmid primers (fig 3c). The RT-PCR products were excised from the agarose gel and directly sequenced. The plasmid bearing the mutant *POU1F1* yielded the two aberrant splicing products corresponding to the partial and total skipping of exon 3 detected in the patient's lymphocytes whereas the wild-type yielded only the normal isoform. A faint band of the same electrophoretic mobility as the wild type cDNA was detected also among the mutant products, but it

was too weak for sequencing. No additional alternative splicing fragment of the mutant allele was observed in vitro with respect to those observed in the lymphocytes.

DISCUSSION

We here report a novel homozygous mutation in the *POU1F1* gene at position -3 of the acceptor site of IVS2 in a female patient affected with hypopituitarism born to consanguineous parents. The patient presented with failure to thrive at three months of age and GH-, TSH- and PRL- deficiency. Cranial MRI revealed pituitary hypoplasia a feature that is often associated to *POU1F1* gene mutations [31,32]. The early diagnosis and replacement treatment with L-thyroxine and recombinant GH assured a completely normal outcome. The patient showed spontaneous pubertal development and reached a near final height that is already above her target height. Her IQ showed a normal intellectual outcome contrarily to what observed in cases in which the diagnosis of central hypothyroidism has been made later in life leading to severe psychomotor impairment [28].

As demonstrated from the lymphocyte mRNA analysis and from the in vitro splicing experiment the mutation found in this patient leads to the production of two aberrantly spliced products. The first is a mRNA missing the first 71 bases of exon 3 due to a partial skipping of this exon, putatively encoding a protein truncated at amino acid 82 (fig1b). Similarly to other mutant *POU1F1* alleles, such as Lys145ter [26], this mRNA isoform, if not degraded as a consequence of nonsense mediated mRNA decay [33], is expected to encode a protein completely lacking the DNA binding domain and thus unable to bind the target gene promoters. The second aberrant mRNA isoform produced by the IVS2-3insA allele is an exon 3–lacking mRNA which maintains the correct reading frame, encoding a putative POU1F1 protein deleted from residue 72 to 146 (fig.1c). This mutant molecule lacks a part of the transactivation domain and a region, the first α-helix of the POU-S homeodomain, that participates to the recruitment of cofactors in the transcriptional complex [34]. Thus, the mRNA lacking exon 3, might give rise to a POU1F1 molecule that retains the ability to bind DNA but is defective in the transcriptional activation of target genes.

It is thus likely that none of the mRNA isoforms present in the patient encodes a protein capable of promoting gene transcription.

Interestingly all the family members carrying the heterozygous mutation showed a prolactin level below the normal range indicating a mild endocrine dysfunction. Prolactin deficiency has been reported to result in the lack of puerperal lactogenesis and, more rarely, in delayed puberty and subfertility [35,36]. None of these clinical symptoms were observed in the heterozygous subjects, apart from the mother's hypogalactorrhea after the third pregnancy. Our data confirm the observation reported by Hashimoto et al. [26] that described a similar mild phenotype in the heterozygous parents of a CPHD patient homozygous for the Lys145ter mutation. They both showed PRL and TSH level on the lower side of the normal range and the mother had hypogalactorrhea. The heterozygous mother described by Pellegrini-Bouiller et al. [15], carrying the F135C mutation, was able to achieve normal lactation despite low normal PRL levels. In addition, she had short stature (149 cm) and low normal IGF-I levels, whereas, in our family the heterozygous subjects showed normal stature and normal IGF-I levels. To date no other heterozygous relatives of patients homozygous for *POU1F1* mutations have been investigated at the hormonal level and thus the phenotypic consequences for carriers of only one functional allele are not yet well defined. The mild endocrine dysfunction reported in the heterozygotes for *POU1F1* mutant alleles that have been evaluated so far suggests that one normal allele might not be able to assure a completely adequate pituitary function and that these subjects may require a careful follow-up.

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TABLE 1. Hormonal details of the patient's family members.

Subject	PRL (ng/ml)	TSH (mU/l)	FT3 (pg/ml)	FT4 (ng/dl)	IGF-I (µg/L)	ACTH (pg/ml)	Cortisol (µg/dl)
Mother (I-1)	**3.0**	1.7	3.2	1.4	280	11.5	8.4
Father (I-2)	**2.2**	2.0	3.0	1.2	278	13.0	8.1
Sister (II-1)	**3.5**	1.5	3.8	1.4	394	10.0	13.3
Sister (II-3)	10	3.2	4.5	1.3	250	12.8	12.7

Normal ranges: PRL females 5-25 ng/ml, males 5-15 ng/ml; TSH 0.3-4.2 mU/l; FT3 2.0-4.4 pg/ml; FT4 0.9-1.7 ng/dl; IGF-I 11-18 yrs 180-780 µg/L, adults 90-280 µg/L; ACTH 10-130 pg/ml; cortisol 5-20 µg/dl
Below-normal values are bolded.

LEGENDS TO FIGURES

FIGURE 1

Schematic representation of (a) the POU1F1 molecule encoded by the wild-type mRNA and (b and c) the two putative POU1F1 molecules encoded by the two aberrant mRNA isoforms resulting from the IVS2-3insA mutation. The two DNA binding (POU-specific and POU-homeodomain) and the transactivation domains are indicated with the respective amino acid positions (according to ref.3). The 4 α-helices of the POU-S and the 3 α-helices of the POU-H domain are numbered. The aberrant products are b) the product encoded by the partially skipped mRNA lacking the first 71 bases of exon 3: the protein is truncated at amino acid 82. c) the product encoded by the mRNA lacking exon 3: the protein is deleted of part of the transactivation domain and of the first α-helix of the POU-S homeodomain, from residue 72 to 146.

FIGURE 2

Pedigree of the CPHD family. The two parents (I-1 and I-2) are second degree cousins. The affected subject is indicated by a black symbols . + wild-type allele; - IVS2-3insA mutated allele.

FIGURE 3

a) RT-PCR performed on lymphocyte mRNA of all family members. Each individual is indicated with the symbol reported in the pedigree of fig.2. All the visible bands were extracted from the gel and sequenced. The scheme of each product is reported on the right of the molecular weight of the corresponding band. The nested PCR primer positions are indicated by arrows. b) Schematic representation of the constructs used for the in vitro splicing analysis. The arrows indicate the primer position within the pSPL3 exons (black boxes). *POU1F1* exon3 is hatched. c) RT-PCR amplification using primers designed in pSPL3 exons, as indicated in b, from total RNA of COS7 cells transiently transfected with the *POU1F1* wild-type (lane 2) and the IVS2-3insA mutant allele (lane 3) constructs.

FIG1

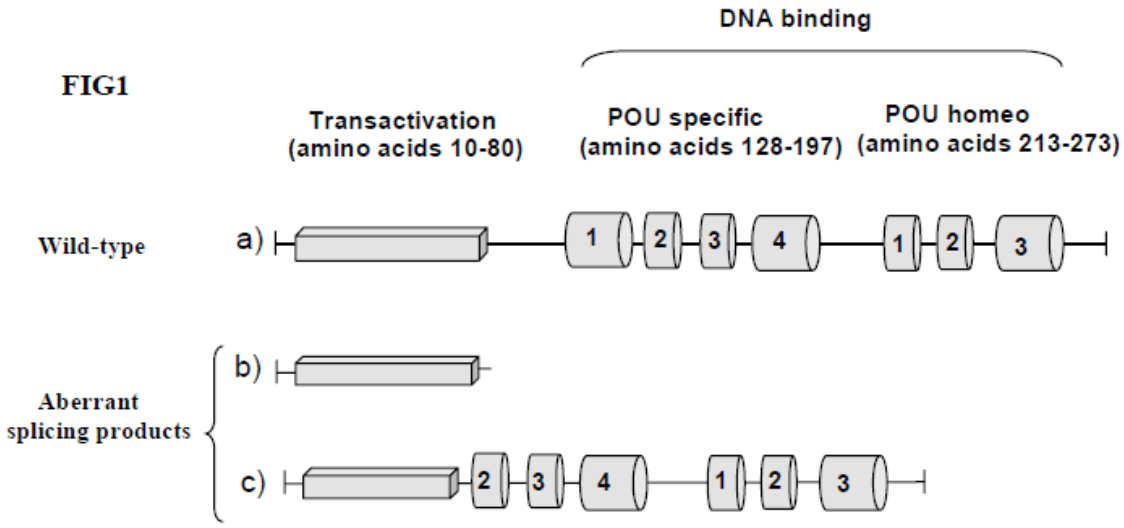
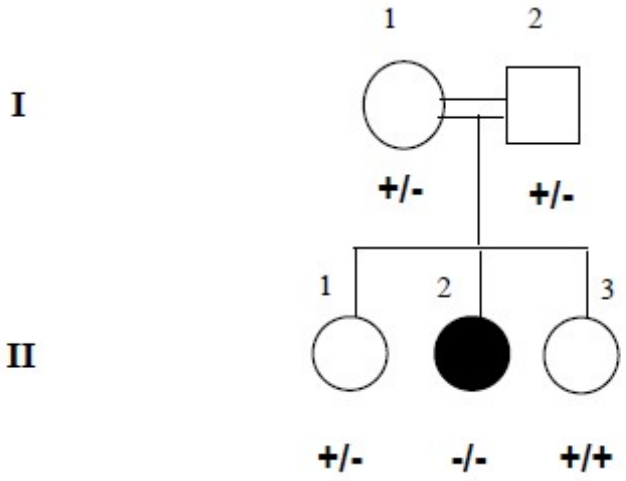

DNA binding
Transactivation
(amino acids 10-80)
POU specific
(amino acids 128-197)
POU homeo
(amino acids 213-273)
Wild-type
a)
1
2
3
4
1
2
3
Aberrant
splicing products
b)
c)
2
3
4
1
2
3

Fig. 2


1
2
I
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1
2
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II
+/-
-/-
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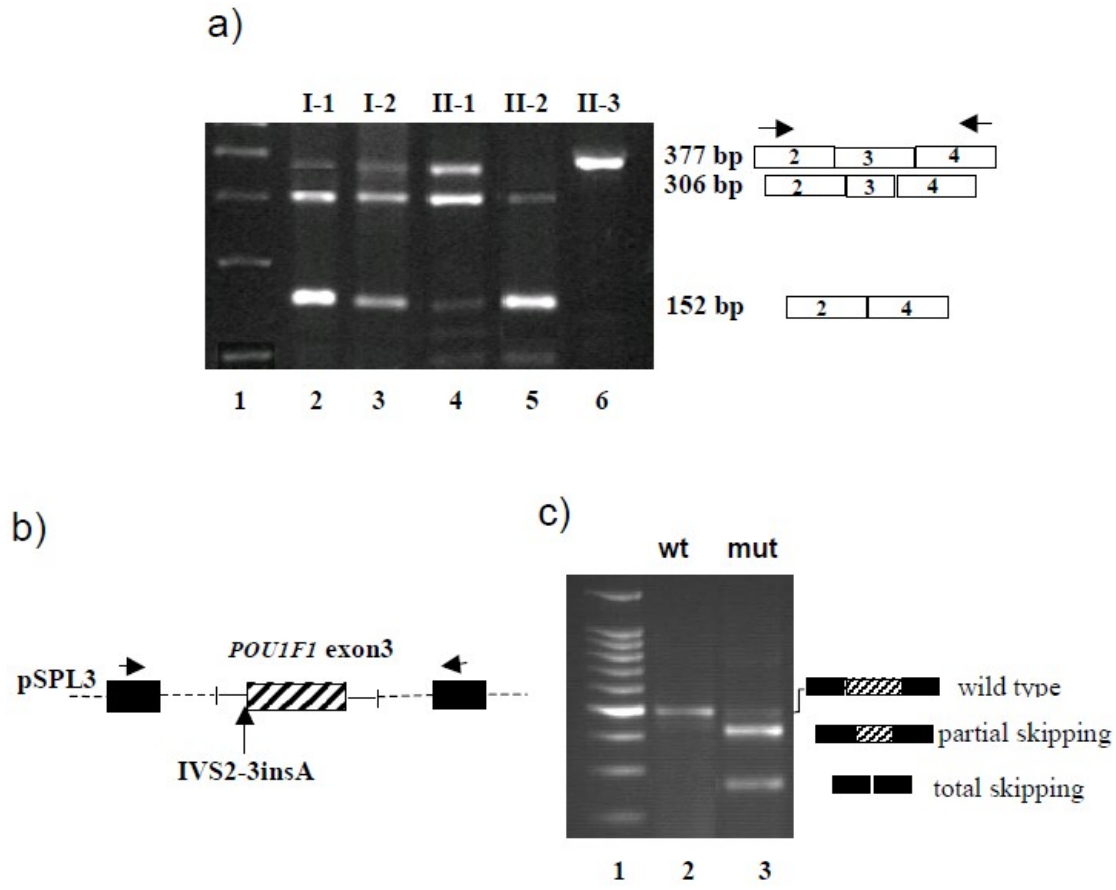
Fig 3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I-1
I-2
II-1
II-2
II-3
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306 bp
152 bp
2
3
4
1
2
3
4
5
6
b)
pSPL3
POU1F1 exon3
IVS2-3insA
c)
wt
mut
wild type
partial skipping
total skipping
1
2
3

7.3 A new case of familial nonautoimmune hyperthyroidism caused by the M463V mutation in the TSH receptor with anticipation of the disease across generations: a possible role of iodine supplementation

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A New Case of Familial Nonautoimmune Hyperthyroidism Caused by the M463V Mutation in the TSH Receptor with Anticipation of the Disease Across Generations: A Possible Role of Iodine Supplementation

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Objective: Hereditary (familial) nonautoimmune hyperthyroidism (FNAH) is caused by activating thyroid-stimulating hormone (thyrotropin) receptor (TSHR) germline mutations. We describe a family with recurrent thyrotoxicosis and goiter across three generations, including an 8-year-old girl. *Main outcome*: Sequences of the TSHR gene in the index patient, her father, her paternal grandmother, and a paternal uncle demonstrated the presence of an identical germline TSHR mutation. The mutation was heterozygous and determined the substitution of valine for methionine (codon 463; ATG→GTG) in the second transmembrane domain of the TSHR in all the affected patients, but in none of the unaffected family members. *Conclusions*: We compared the clinical presentation of FNAH in the family reported by us with the other cases harboring the same mutation reported in the literature. This analysis revealed high variability in the phenotypical expression of the disease. In the family reported by us, we also observed a clear anticipation of the onset of the disease across generations, and we discussed whether such a phenomenon can be the consequence of the increased iodine supplementation in the area where the family lives.

Introduction

FAMILIAL NONAUTOIMMUNE HYPERTHYROIDISM (FNAH) or hereditary toxic thyroid hyperplasia is a clinical entity originally described by Thomas *et al.* in 1982 (1). This condition is clinically characterized by thyroid autonomy in two or more generations with a variable age of onset (from infancy to adulthood), as well as frequent relapses of hyperthyroidism after thyrostatic therapy withdrawal or partial thyroidectomy. Thyroid-stimulating hormone (thyrotropin) receptor (TSHR) antibodies are always absent (2). Thyroid ablation by total thyroidectomy or radiotherapy is the only treatment able to prevent relapses of the disease.

So far, 23 cases of nonautoimmune hyperthyroidism due to 18 different activating germline mutations of the TSHR have been identified (1,3–21).

Since the condition is dominantly autosomal inherited, molecular diagnostics and genetic counseling are advocated in the affected families.

In the present work, we describe the clinical and molecular findings in the second Italian family with FNAH harboring the Met463Val TSHR germline mutation. We also compare the phenotype of the family reported by us with the clinical characteristics of the three other families with the same mutation previously reported (12,17,22).

Case Report

An 8-year-old girl (III-1, Fig. 1) came to our clinic with a 2-year history of tachycardia and tremors. She was born after 40 weeks of gestation by cesarean section for podalic presentation with a birth weight of 2.500 kg. Antenatal and postnatal history was uneventful. Her neurodevelopment was within normal limits. Physical examination revealed a very slight enlargement of the thyroid and her eyes were mildly prominent. Her heart rate was 120 bpm and blood pressure was 120/60 mmHg. Her weight was normal, but her height was above the 95° centile and above the parental height.

Thyroid function tests revealed increased levels of free triiodothyronine and free thyroxine with a suppressed TSH in the absence of antithyroglobulin (anti-Tg), antithyroid peroxidase (anti-TPO), and anti-TSHR antibodies. Thyroid ultrasound showed a slight enlargement of the gland (5.7 mL), with diffuse hypoechogenicity and without nodules. The girl

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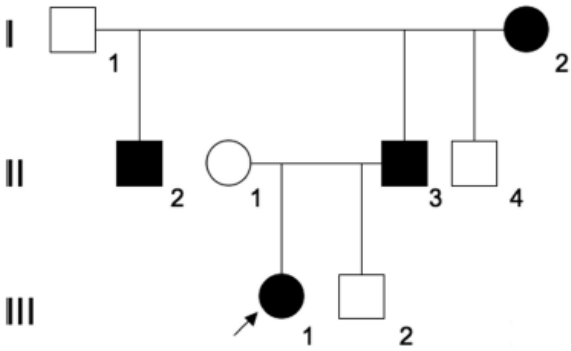

FIG. 1. Pedigree of the studied family. In black are indicated the affected members of the family. The arrow marks the proband.

was started on methimazole (MMI) at a dosage of 0.4 mg/kg/die, with clinical improvement, reduction of the size of the thyroid gland, and reduction of circulating free thyroid hormones levels.

The family history of the patient was positive for nonautoimmune hyperthyroidism, which was present in the father, in a paternal uncle, and in the paternal grandmother (the pedigree of the family is shown in Fig. 1). The father (II-3, Fig. 1) was diagnosed as hyperthyroid at the age of 18 years; anti-Tg, anti-TPO, and anti-TSHR antibodies were all negative. He was unsuccessfully treated with MMI for several years and underwent a near-total thyroidectomy at the age of 35. The histology of the gland indicated a multinodular goiter with signs of hyperfunction. Since thyroidectomy, his thyroid function tests have been within the normal range without any treatment.

The paternal uncle (II-2, Fig. 1) was diagnosed as hyperthyroid with multinodular goiter at 27 years of age. He had no anti-Tg, anti-TPO, or anti-TSHR antibodies. He was treated with MMI for 13 years without full success. When he was 40 years, he underwent total thyroidectomy. Since then, he has been treated with substitutive doses of levothyroxine. The grandmother (I-2, Fig. 1) had been diagnosed with hyperthyroidism and goiter when she was 30 years old. Anti-Tg, anti-TPO, and anti-TSHR antibodies were negative. She never developed thyroid nodules and surgery was not necessary, since she has always been successfully treated with MMI.

Materials and Methods

Informed consent was obtained from all subjects included in the study. Genomic DNA was extracted from peripheral blood leucocytes of eight family members indicated in Figure 1. All the exons of the TSHR gene were amplified by polymerase chain reaction (PCR) primers, and conditions will be provided upon request. PCR products were purified with QIAquick PCR Purification Kit and directly sequenced using a 377 ABI sequencer.

Results

The sequence of exon 10 in the propositus (III-1, Fig. 1) showed the presence of a heterozygous mutation from adenine to guanine (ATG→GTG) in the first base of the triplet encoding for the amino acid at position 463 of the TSHR (Fig. 2). The mutation results in a change from methionine to valine (Met463Val). The Met463Val mutation was also found in the father (II-3, Fig. 1), in a paternal uncle (II-2, Fig. 1), and in the grandmother (I-2, Fig. 1) of the proband (all affected with toxic goiter). No mutations in the coding region of the TSHR gene have been found in any of the unaffected members of the family.

Discussion

Constitutive activation of TSHR is the cause of the autosomal-dominant nonautoimmune hyperthyroidism. This is a rare disorder with an incidence of less than 1% in patients with juvenile hyperthyroidism, and with a frequency of 6% in patients with thyrotoxicosis without thyroid antibodies (16). Most of the activating germline mutations have been identified in exon 10 of the TSHR gene, encoding for the transmembrane and intracellular portions of the TSHR. So far, only the Ser281Asn mutation occurred in the exon 9 (13). The majority of germline mutants (44%) have been identified in the sixth and seventh transmembrane helixes (23).

Here we describe an Italian family with nonautoimmune hyperthyroidism occurring over three generations. The disease is caused by a Met463Val mutation in the second transmembrane domain of the TSHR.

The mutation has been previously reported (12,17,22) and functionally characterized by Fuhrer *et al.* (12). The Met463Val mutant shows an increased binding affinity for TSH and cAMP production when compared to the wild-type TSHR, but has no effects on the activation of the inositol-phosphate pathway, which has been found to be stimulated by other somatic TSHR mutations (24).

The Met463Val has been previously identified in three families: a Welsh (12), an Italian (22), and a Thai (17). It is worth noting that, despite the same genetic alteration, there are relevant differences in the clinical presentation between the three families previously described as well as in the one that we report here.

One of the differences is related to the age of onset of hyperthyroidism in the families with the Met463Val muta-

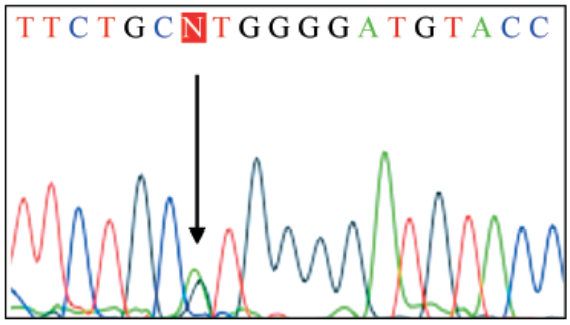

FIG. 2. Partial sequence of the exon 10 of the thyroid-stimulating hormone receptor (TSHR) of the proband. The arrow indicates the heterozygous mutation from adenine to guanine (ATG→GTG) in the first base of the triplet encoding for the amino acid at position 463 of the TSHR. Double pick was not present in the sequence of the unaffected members of the family.

tion. In fact, while Arturi *et al.* (22) reported that hyperthyroidism occurred after puberty, between 11 and 18 years of age, in the families of Fuhrer *et al.* (12) and Lee *et al.* (17), the age of onset was more spanning, ranging from 4 to 21 years. In the family reported by us, the age of onset of hyperthyroidism is widely spread (grandmother: 30 years, uncle: 27 years, father: 18 years, and proband: 8 years) with anticipation across generations. Anticipation across generations was already reported by Lee *et al.* (17) and Fuhrer *et al.*; however, in the family reported by us it is more evident.

Second, the evolution of thyroid hyperplasia in the family reported by us is peculiar. In the family reported by Fuhrer *et al.* (12), the manifestations of hyperthyroidism precede the development of goiter, suggesting that the initial manifestations of the constitutive activation of TSHR are exclusively functional. In other cases of FNAH (13), it has been proposed that a longer period of time is required for the appearance of goiter. In the family reported by Arturi *et al.* (22), the goiter always preceded the onset of hyperfunction and the clinical signs of hyperthyroidism are mild in all affected members.

In our proband, the signs of hyperthyroidism were responsible for the diagnosis and there was only a slight thyroid enlargement. These are contrary to what happened in the other affected members of the family, where hyperthyroidism followed the goiter appearance.

Third, the relapse time of hyperthyroidism after partial thyroidectomy was different among the families. In the family reported by Arturi *et al.*, (22) the relapse of hyperthyroidism after thyroidectomy ranged from 6 months to 17 years. In the family reported by Fuhrer *et al.*, (12) the index patient's aunt relapsed shortly after a partial thyroidectomy and the maternal grandmother was euthyroid after thyroid surgery. In the family reported by us, the girl's father, who underwent thyroidectomy earlier than his affected brother, since the surgery, about 20 years ago, has been euthyroid without any further treatment.

Finally, in the family reported by us, both affected males, who underwent thyroidectomy, had a multinodular toxic goiter while the index patient's grandmother had a diffuse toxic goiter and the proband had thyrotoxicosis with a slight diffuse enlargement of thyroid. So it should seem that there is, in the family reported by us, a close relationship between thyroid morphology and the gender of the patients.

The variability in the clinical phenotype of the family reported by us, despite the same genetic alteration in the TSHR gene, confirms the hypothesis that other factors can play a role in the appearance of the phenotype modulating the effects of the mutations on the cAMP pathway by complex interactions (25). It has been proposed that the different expression of mutations may reflect differences in the genetic background of the affected individuals, as well as differences in environmental factors, such as goitrogens or iodine intake in the diet (26,27). As described by Alberti *et al.* (5), dietary iodine intake may contribute to the wide range in age of onset of symptoms reported in families harboring the same activating germline TSHR mutations.

In the family we reported, the presence of a general tendency toward a younger age presentation of the disease in subsequent generations could be linked to an increased exposure to dietary iodine. During the 1980s, when the grandmother of our proband was diagnosed hyperthyroid and during the 1990s, when the father and the uncle of the patient underwent thyroidectomy, the mean urinary excretion of iodine in the province of Napoli, where the family comes from, was 85.8 mcg/L (28). Despite the fact that the prophylaxis with iodized salt was started only very recently, several factors may have increased the iodine supplementation in the diet, such as silent prophylaxis (29). These factors may have determined an increase in urinary iodine excretion to 93 mcg/L (IEOS-CNR, Italy: Programma per l'eradicazione del gozzo endemico e dei disordini da carenza iodica nell'Italia Meridionale 1999, unpublished data). Moreover, the anamnesis confirmed that the family reported by us has been using iodized salt for at least the past 10 years. Accordingly, the diagnosis of thyrotoxicosis was reached at 30, 27, and 18 years of age in the older members of the family and at 8 years of age in the proband.

In conclusion, we reported a new family with Met463Val germline mutation in the TSHR gene causing FNAH. The mutation that we observed has already been reported and, including in the family reported by us, it seems to be the most frequent cause of FNAH not only in the Italian population but also all over the world.

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APPENDIX I

LIST OF COMMUNICATIONS TO NATIONAL AND INTERNATIONAL MEETINGS

AM Ferrara, G Rossi, S Captano, G Del Prete, **D Capalbo**, V Esposito, G Fenzi, M Salerno, PE Macchia, FAMILIAL HYPERTHYROIDISM CAUSED BY AN ACTIVATING MUTATION IN THE TSH RECEPTRO GENE, XXVI Giornate Endocrinologiche Pisane, Pisa, 8/10 Giugno 2006

C. Camacho-Hubner; L. M Hernandez; L. Martin; **D. Capalbo**; J.C. Blair; C. E. Martinelli; M.O. Savage. STUDIES OF ACID LABILE SUBUNIT IN CHILDREN WITH SEVERE IDIOPATHIC SHORT STATURE. 46nd Annual Meeting of the European Society for Paediatric Endocrinology, Helsinki, Finland, 27-30 June, 2007. Horm. Res. 2007; 68 (S1)

D. Capalbo; A. Ciao; D. M. Mattiacci; S. Boemio; M. Ferrara; P. Macchia; M. Salerno. FAMILIAL HYPERTHYROIDISM CAUSED BY AN ACTIVATING MUTATION IN THE TSH RECEPTOR GENE. 46nd Annual Meeting of the European Society for Paediatric Endocrinology, Helsinki, Finland, 27-30 June, 2007. Horm. Res. 2007; 68 (S1): 108

A. Lo Vecchio; **D. Capalbo**; G. Montesano; S. Muzzica; V. Farina; M. Salerno. ECHOCARDIOGRAPHIC ASSESSMENT OF CARDIAC FUNCTION IN CHILDREN AFFECTED BY GH DEFICIENCY AND AFTER 2 YERAS OF GH TREATMENT: A CASE-CONTROL PROSPECTIVE STUDY. 46nd Annual Meeting of the European Society for Paediatric Endocrinology, Helsinki, Finland, 27-30 June, 2007. Horm. Res. 2007; 68 (S1): 157.

D. Capalbo, A. Lo Vecchio, A. Palladino, D.M. Mattiacci, A. Ciao, V. Farina, M. Salerno**.** FUNZIONE CARDIACA IN BAMBINI AFFETTI DA DEFICIT DI GH: EFFETTO NDELLA TERAPIA SOSTITUTIVA. XVI Congresso Nazionale SIEDP Parma, 11-13 Ottobre 2007.

D. Capalbo, L. Mazzanti, C. Di Somma, F. Egger, M. Salerno, Y. Leitner, G. Radetti. RISCHIO CARDIOVASCOLARE IN PAZIENTI AFFETTE DA SINDROME DI TURNER. XVI Congresso Nazionale SIEDP Parma, 11-13 Ottobre 2007.

D. Capalbo, S. Muzzica, M. Cerbone, N. Improda, A.M. Ferrara, P.E. Macchia, M. Salerno. IPERTIROIDISMO FAMILIARE CAUSTAO DA MUTAZIONE DEL RECETTORE DEL TSH. XVI Congresso Nazionale SIEDP Parma, 11-13 Ottobre 2007.

I Russo, S. Amorosi, L. Vitiello, D. Capalbo, T. Broccoletti, M. Salerno, C. Pignata ANALISI DELL'ALTERAZIONE DEI MECCANISMI DI TOLLERANZA PERIFERICA ASSOCIATA AD ALTA VARIABILITA' FENOTIPICA. Incontri pediatrici Normanni. XI Congresso Internazionale. Aversa, 22-24 Novembre 2007

D. Capalbo, A Lo Vecchio, S. Muzzica, A Palladino, L. Spinelli, A. Colao, V. Farina, M. Salerno. CARDIAC FUNCTION AND MORPHOLOGY IN CHILDREN WITH GH DEFICINECY BEFORE AND AFTER TWO YEARS OF GH REPLACEMENT THERAPY. 90th Annual Meeting, The Endocrine Society, San Francisco, June 15-18, 2008.

D. Capalbo, D.M. Mattiacci, S. Muzzica, G. Montesano, A. Palladino, M. Cerbone, C. Mainolfi, M. Salerno. EFFECTS OF GH THERAPY ON FINAL HEIGHT AND BONE MINERAL DENSITY IN TURNER SYNDROME. 47th Annual Meeting of the European Society for Paediatric Endocrinology (ESPE), Instanbul, Turkey, September 20-23, 2008

Capalbo D, Amorosi S, Palladino A, Mattiacci D, Vitiello L, Betterle C, Pignata C, Salerno M. AMPIA VARIABILITA' FENOTPICA NELLA SINDROME APECED: STUDIO DEI MECCANISMI DI TOLLERANZA IMMUNOLOGICA PERIFERICA, Giornata di Ricerca del Dipartimento di Pediatria, Università degli studi di Napoli Federico II, 2009

Donatella Capalbo, Stefania Amorosi, Cinzia Mazza, , Corrado Betterle, , Angela Palladino, , Alessandro Ciao, Claudio Pignata, and Mariacarolina Salerno, HIGH INTRAFAMILIAR VARIABILITY IN APECED: STUDY OF THE PERIPHERAL TOLERANCE. ESPE 2009 , New York

Donatella Capalbo, Cinzia Mazza, Roberta Giordano,Giovanna Montesano, Emanuela Arvat, Corrado Betterle, Claudio Pignata, and Mariacarolina Salerno, HIGH PREVALENCE OF EXON 1 MUTATIONS IN APECED PATIENTS FROM CAMPANIA, A REGION OF SOUTHERN ITALY , ESPE 2009, New York

EFFETTO DEL DEFICIT DI ORMONE DELLA CRESCITA E DELLA TERAPIA SOSTITUTIVA CON GH SUI LIVELLI EMATICI DI FERRITINA E FIBRINOGENO IN ETÀ PEDIATRICA. G. Montesano, C. Palladino, A. Ciao, M. Cerbone, S. Muzzica, **D. Capalbo**,M. Salerno;Giornate Scientifiche Polo delle Scienze e Tecnologie per la vita, Università Degli Studi di Napoli Federico II

RUOLO DEI TRIGGERS AMBIENTALI E DEI MECCANISMI DI TOLLERANZA PERIFERICA NELLA VARIABILITA' FENOTIPICA DELL'APECED. **D Capalbo**, S. Amorosi, G. Giordano, F. Maio, M. Cerbone, L. Vitiello, C. Pignata, M. Salerno. XVII Congresso Nazionale SIEDP Napoli 4-7 Novembre 2009

PATTERN GENETICO ED ESPRESSIONE FENOTIPICA IN PAZIENTI CAMPANI AFFETTI DA APECED E NEI LORO FAMILIARI. **D. Capalbo**, C. Mazza, R. Giordano, E. Arvat, C. Betterle, C. Pignata, M. Salerno. XVII Congresso Nazionale SIEDP Napoli 4-7 Novembre 2009

VALUTAZIONE PROSPETTICA DELLA CRESCITA E DEL PROFILO LIPIDICO IN BAMBINI CON IPOTIROIDISMO SUBCLINICO. M. Cerbone**, D. Capalbo**, S. Muzzica, D. Cioffi, N. Improda, C. de Leonibus, V. Bove, M. Salerno. XVII Congresso Nazionale SIEDP Napoli 4-7 Novembre 2009

LIVELLI DI LIPIDI, ADIPOCITOCHINE, E FIBRINOGENO IN BAMBINI AFFETTI DA DEFICIT DI GH PRIMA E DOPO TERAPIA SOSTITUTIVA. **D. Capalbo**, G Mattace Raso, V. Bove, R Meli, M. Salerno XVII Congresso Nazionale SIEDP Napoli 4-7 Novembre 2009

M. Salerno, V. Esposito, V. Farina, G. Radetti, A. Umbaldo, **D. Capalbo**, L. Spinelli, S. Muzzica, G. Lombardi, A. Colao IMPROVEMENT OF CARDIAC PERFORMANCE AND CARDIOVASCULAR RISK FACTORS IN CHILDREN WITH GH DEFICIENCY AFTER TWO YEARS OF GH REPLACEMENT THERAPY: AN OBSERVATIONAL, OPEN, PROSPECTIVE, CASE-CONTROL STUDY. J Clin Endocrinol Metab. 2006 Apr; 91(4):1288-1295.

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APPENDIX II

GRANT PROPOSAL

1. Progetto PRIN 2008: " Studio dei fattori funzionali e genetici coinvolti nella modulazione dell'espressione fenotipica della sindrome poliendocrina autoimmune associata a mutazione del gene AIRE"
2. Stesura del Progetto Regionale Legge 5 dal titolo: Identificazione di marcatori precoci di rischio metabolico e cardiovascolare in bambini affetti da Deficit di Ormone della crescita"
3. AIP Application 2009, Clinical and molecular characterization of pediatric patients affected with APECED: identification of functional and genetic factors influencing the phenotype expression of the disease.