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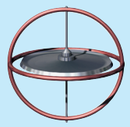

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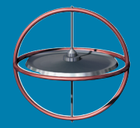

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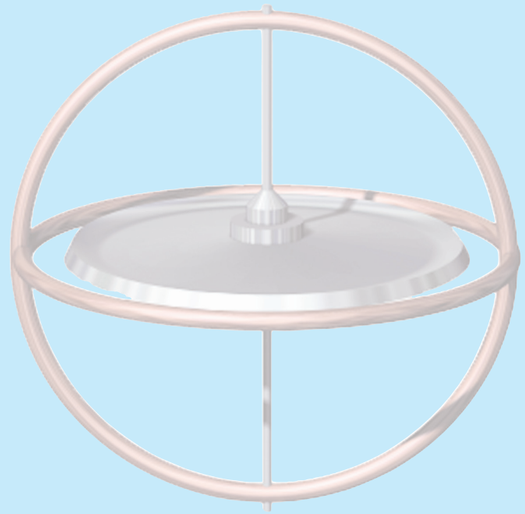

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Review

Endocrine sequelae of immune checkpoint inhibitors

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ABSTRACT

Cancer immunotherapy has introduced a novel class of drugs known as immune checkpoint inhibitors (ICIs). They enhance antitumour immunity by blocking negative regulators (checkpoints) of T cell function that exist on both immune and tumour cells. ICIs targeting CTLA-4 and PD-1/PDL-1 have dramatically changed the outcome of patients with several advanced-stage malignancies but they may lead to a variety of inflammatory toxicities and autoimmune consequences. The main endocrine immune-related adverse events (IRAEs) include hypophysitis, primary thyroid dysfunction, adrenalitis and type 1 diabetes mellitus. In general, the management of endocrine IRAEs requires assessment of their severity, in moderate or severe cases interruption of the checkpoint inhibitor and use of corticosteroid or alternative immunosuppression and appropriate hormone replacement or treatment when necessary.

Key words: Anti-CTLA4, Anti-PD1, Anti-PDL1, Autoimmune consequences, Immune checkpoint inhibitors

INTRODUCTION

Cancer immunotherapy has introduced a novel class of drugs known as immune checkpoint inhibitors (ICIs). They enhance antitumour immunity by blocking negative regulators (checkpoints) of T cell function that exist on both immune and tumour cells. The first agent was approved in 2011 and since then a few of them have been evaluated in clinical trials for the management of advanced neoplasias, such as metastatic melanoma, non-small cell lung cancer, advanced renal cell carcinoma, head and neck cancer and lymphoma (Table 1).[1-5] However, because of their mechanism of action these agents can lead to a variety of inflammatory toxicities and autoimmune consequences that can affect the gastrointestinal tract, skin, liver and endocrine system and are hence described as immune-related adverse events (IRAEs). The endocrine immune-related adverse events include hypophysitis, primary thyroid dysfunction, adrenalitis and type 1 diabetes mellitus which present with variable degrees of severity[6-11,38] (Table 2) and incidence rates[47] (Table 3).

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PATHOGENIC MECHANISMS

Tumour antigens are presented by dendritic cells

Table 1. FDA approved immune check point inhibitors

Product Generic name (Brand name)	Indication for the treatment of patients with cancer
Anti-CTLA4	
Ipilimumab (Yervoy)	cutaneous melanoma unresectable or metastatic melanoma
Tremelimumab	malignant mesothelioma
Anti-PDL1	
Atezolizumab (Tecentriq)	locally advanced or metastatic urothelial carcinoma metastatic squamous cell carcinoma of the head and neck
Avelumab (Bavencio)	locally advanced or metastatic urothelial carcinoma metastatic Merkel cell carcinoma (MCC)
Durvalumab (Imfinzi)	locally advanced or metastatic urothelial carcinoma metastatic Merkel cell carcinoma (MCC)
Anti-PD1	
Nivolumab (Opdivo)	locally advanced or metastatic urothelial carcinoma recurrent or metastatic squamous cell carcinoma of the head and neck renal cell carcinoma unresected or metastatic melanoma non-small cell lung cancer classical Hodgkin lymphoma that has relapsed or progressed after autologous hematopoietic stem cell transplantation
Pembrolizumab (Keytruda)	adult and pediatric patients with unresectable or metastatic, microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) solid tumors locally advanced or metastatic urothelial carcinoma refractory classical Hodgkin lymphoma recurrent or metastatic head and neck squamous cell carcinoma unresectable or metastatic melanoma. metastatic non-small cell lung cancer
Nivolumab + ipilimumab	BRAF V600 wild-type, unresectable or metastatic melanoma

to cytotoxic T lymphocytes (CTLs). The activation of T cells is regulated by stimulatory and inhibitory signals that maintain the balance between appropriate recognition and destruction of tumour cells and inappropriate immune overstimulation, which damages normal healthy tissue. This is referred to as the cancer-immunity cycle.[12] T cell-mediated inhibitory signalling pathways permit tolerance to tumour antigens. Although there are many T cell checkpoints, antibodies against cytotoxic T-lymphocyte antigen 4 (CTLA4), programmed cell death 1 (PD1) and programmed cell death 1 ligand 1 (PDL1) have been most extensively evaluated in the clinical setting. The available data refer to ipilimumab and tremelimumab, both CTLA4-abs, nivolumab, pembrolizumab and pidilizumab, all anti-PD1 abs and atezolizumab an anti-PDL1 agent.

Anti-CTLA4

CD28 on the T cell surface binds to the B7 co-stimulatory ligand on antigen-presenting cells to prime and activate the T cell so as to destroy the cancer cells. CTLA4, which is a CD28 homolog, binds with higher affinity to B7 and can compete with CD28 to inhibit T cell activity. This process prevents the T cell from killing the cancer cells. Antibodies to CTLA4 prevent B7 binding and enable upregulation of T cell activity, activation of mitogen-activated protein kinase which results in the formation of activator protein 1 (AP1) complex and induction of IL-2 cytokines which mediate T cell growth.[13-15]

Anti-PD1 and anti-PDL1

The PD1 (programmed cell death - 1) receptor (also known as CD279) is expressed on the surface of

Table 2. Classification of endocrine adverse events and diagnostic criteria

	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Hypophysitis	Asymptomatic or mild symptoms (mild fatigue, anorexia, no headache); clinical or diagnostic observations only; intervention not indicated	Moderate (i.e. fatigue, mood alteration, headache but without visual disturbances) minimal, local or noninvasive intervention indicated; limiting age appropriate instrumental ADL	Severe or medically significant but not immediately life-threatening hospitalization or prolongation of existing hospitalization indicated; disabling; limiting self care	Life-threatening consequences; urgent intervention indicated	Death
Hypothyroidism	Asymptomatic; clinical or diagnostic observations only; intervention not indicated	Symptomatic; thyroid replacement indicated; limiting instrumental ADL	Severe symptoms; limiting self care; ADL; hospitalization indicated	Life-threatening consequences; urgent intervention indicated	Death
Hyperthyroidism	Asymptomatic; clinical or diagnostic observations only; intervention not indicated	Symptomatic; thyroid suppression therapy indicated; limiting instrumental ADL	Severe symptoms; limiting self care; ADL; hospitalization indicated	Life-threatening consequences; urgent intervention indicated	Death
Adrenalitis	Asymptomatic; clinical or diagnostic observations only; intervention not indicated	Moderate symptoms; medical intervention indicated	Severe symptoms; hospitalization indicated	Life-threatening consequences; urgent intervention indicated	Death

The National Cancer Institute has recommended that adverse events on patients with cancer chemotherapy be graded as per the Common Terminology Criteria for Adverse Events (CTCAE) Version 4.03. (U.S. Department of Health and Human Services, National Institutes of Health, National Cancer Institute. Bethesda, MD, USA). Although the published guidelines do not specifically comment on hypophysitis, the toxicity grading structure has been applied to both pituitary function and other endocrine organs.

Table 3. Incidence rates of IRAEs endocrine sequelae. (data from Barroso-Sousa et al[47])

	Hypophysitis incidence rate (95% CI)	Hypothyroidism incidence rate (95% CI)	Hyperthyroidism incidence rate (95% CI)	Primary adrenal insufficiency N of pts with PAI/ total n of pts (%)*	N of pts with DM1/ total n of pts (%)
Anti-CTLA4	0.0 (0.0-6.7) - 6.5 (1.4-17.9)	0.0 (0.0-45.9) - 15.2 (6.3-28.9)	0.0 (0.0-45.9) - 2.3 (0.9-5.0)	43/5831 (0.7%)**	13/5831 (0.2%)***
Anti-PDL1	0.0 (0.0-5.9) - 3.0 (0.1-15.8)	0.0 (0.0-1.2) - 5.6 (2.5-10.8)	0.0 (0.0-1.2) - 0.7 (0.2-1.8)		
Anti-PD1		0.0 (0.0-30.8) - 40.0 (19.1-63.9)	0.0 (0.0-9.0) - 7.7 (1.6-20.9)		
Combination	3.8 (0.5-13.0) - 11.7 (6.0-20.0)	3.8 (0.5-13.0) - 16.0 (9.2-25.0)	3.8 (0.5-13.0) - 9.9 (6.8-13.8)	11/262 (4.2%)	

*number of patients with primary adrenal insufficiency as adverse event following immune check point inhibitors therapy/total number of patients who received ICIs therapy.
** Refers to pts taking Anti-CTLA4/Anti-PDL1/Anti-PD1; *** Refers to pts taking Anti-CTLA4/Anti-PDL1/Anti-PD1 or combination of them.
PAI: primary adrenal inufficiency; DM1: diabetes mellitus type 1.

activated T cells. Its ligands, PDL1 (B7-H1; CD274) and PDL2 (B7-DC; CD273) are commonly expressed on the surface of antigen-presenting/dendritic cells or macrophages. PD1 and PDL1/PDL2 belong to

the family of immune checkpoint proteins that act as co-inhibitory factors, which can stop or limit the development of the T cell response. PD1/PDL1 interaction forms a biochemical "shield" protecting tumour cells from being destroyed by the immune system. Monoclonal antibodies that target either PD1 or PDL1 can block this binding and boost the immune response against cancer cells.[16,17]

GENERAL MANAGEMENT OF IMMUNE-RELATED ADVERSE EVENTS

In general, treatment of moderate or severe IRAEs requires interruption of the checkpoint inhibitor and the use of corticosteroid or alternative immunosuppression.[18] For grade 2 (moderate) immune-mediated toxicities, treatment with the checkpoint inhibitor should be withheld and not resumed until symptoms or toxicity is grade 1 or less. Corticosteroids (prednisone 0.5mg/kg/day or equivalent) should be started if symptoms do not resolve within a week. For patients experiencing grade 3 or 4 (severe or life-threatening) immune-mediated toxicities, treatment with the checkpoint inhibitor should be permanently discontinued and high doses of corticosteroids (prednisone 1-2mg/kg/day or equivalent) should be given. When symptoms subside to grade 1 or less, steroids can be gradually tapered over at least one month.[18]

If symptoms do not clearly improve, particularly after approximately three days with intravenous steroids, infliximab (5mg/kg) could be administered rather than continuing with a prolonged course of high-dose IV corticosteroids.[18] The need for immunosuppressive therapy to manage IRAEs does not appear to affect the response to checkpoint inhibition with either anti-PD1 antibodies or ipilimumab.[19]

The specific approach to each endocrine IRAE is further discussed in the individual sections below.

ENDOCRINE SEQUELAE

Hypophysitis

Hypophysitis is a rare inflammatory condition of the pituitary gland with an incidence of 1:1,000,000 in the general population. Byun et al estimated that amongst 2,017 ipilimumab treated patients 184 (9.1%) developed hypophysitis, while this consequence occurred only in 8/613 (1.3%) patients on tremelimumab, in 6/929 (0.4%) on nivolumab and in 5/729 (0.68%) on pembrolizumab therapy.[20] A meta-analysis by Abdel-Rahman et al estimated a significantly increased risk of all-grade hypophysitis in patients treated with ICIs versus controls (RR: 22.03, 95% CI: 8.52-56.94).[21]

In contrast, in another meta-analysis of PD1 inhibitors studies the risk of hypophysitis in patients treated with these agents was not significantly elevated compared with controls (RR: 2.32, 95% CI: 0.574-9.40).[22] The authors assumed that this discrepancy could be due to the fact that Abdel-Rahman et al analyzed mainly anti-CTLA4 inhibitors studies (five RCTs evaluating ipilimumab, one evaluating tremelimumab, three for nivolumab and one for pembrolizumab), while all ten RCTs included in their analysis were for PD-1 inhibitors. Notably, PD-1 inhibitors induced a significantly lower risk of hypophysitis when directly compared with ipilimumab (RR: 0.148, 95% CI: 0.043-0.505). This may be explained by the fact that these agents act differently. Anti-CTLA4 therapy can stimulate *de novo* pituitary-reactive effector T cells and also lead to the production of anti-pituitary antibodies and activation of the complement, while anti-PD1/PDL1 therapy can make existing pituitary reactive effector T cells more active. Moreover, the pituitary gland may express CTLA-4, making it a direct target for anti-CTLA-4 antibodies.[23] Of note, the combination of nivolumab/ipilimumab augmented the risk for hypophysitis versus monotherapy with ipilimumab only (RR: 1.94, 95% CI 1.07-3.50).[22]

Hypophysitis usually develops between 5-36 weeks of treatment but late occurrence at 19 months has also been reported.[20,26] Median time to diagnosis of ipilimumab-hypophysitis is nine weeks.[24] Clinical manifestations relate either to sellar compression as the pituitary enlarges (headache, visual defects) or to hormonal disturbance due to autoimmune inflammation of the pituitary (hypotension, nausea, abdominal pain, anorexia, weight loss, temperature intolerance, loss of libido, polyuria, polydipsia). Many of these symptoms are nonspecific and could be attributed either to pituitary dysfunction or to the underlying illness. Late onset of hypopituitarism without a clinically evident acute phase of inflammation has been reported suggesting a low-grade immunological process against the pituitary.[25]

Anterior hypopituitarism is more prevalent than diabetes insipidus, while ACTH and/or TSH deficiency are the most common manifestations.[20,26] Hypogonadotropic hypogonadism and low levels of insulin-like growth factor 1 (IGF1) may also be present.[20] Both high and low levels of prolactin have been described.[24] Older age and male sex may be risk factors for the development of hypophysitis with ICI medications.[27]

It is prudent to perform a baseline hormonal assessment at the initiation of immunotherapy. We suggest that a detailed questionnaire regarding suspicious symptoms for hypophysitis (headache, nausea, weakness, fatigue, hypotension, hypoglycemia) is carried out and measurement of TSH, fT4, morning cortisol (9am), electrolytes, glucose before each cycle is performed, taking into account that both cancer and hypopituitarism may share common symptoms and laboratory results. In the event of compression symptoms (headache, visual defects) and/or clinical suspicion of hypophysitis, then pituitary MRI and a full endocrine work-up (FSH/LH, E2/TESTO, IGF-1, PRL, TSH, fT4, cortisol (9am), ACTH) should be performed (Figure 1).

If morning cortisol is less than 250nmol/l or random cortisol less than 150nmol/l with vague symptoms, then replacement therapy with glucocorticoids should be initiated (Figure 1). In the case of clinical suspicion of adrenal insufficiency or when morning cortisol is <350nmol/l, further dynamic ACTH testing is recommended.

Differential diagnosis should exclude the new occurrence of brain metastases, therefore an MRI is mandatory to rule out this possibility and to check pituitary status. Pituitary morphology may change during the disease course, from mild to moderate diffuse enlargement with homogenous or heterogeneous enhancement after contrast administration and stalk thickening at disease onset, to subsequent atrophy of the gland and finally empty sella. Importantly, a normal MRI does not rule out hypophysitis and management should be based on clinical presentation and hormonal evaluation. Occasionally, changes of pituitary morphology may precede function or biochemical disturbances; this may resolve after 1-8 weeks of glucocorticoid therapy.[27]

The management of hypophysitis primarily involves hormone replacement and consideration of ICIs discontinuation and/or high-dose (immunosuppressive) steroid use. Although congruence between treatment plans is lacking, it is suggested that for patients with grade 1 (mild) hypophysitis, immunotherapy may be continued, while for all other grades of toxicities treatment should be withheld and high-dose systemic steroids (prednisolone 0.5-2mg/kg/day or equivalent), with subsequent tapering to a physiological replacement dose of hydrocortisone or prednisolone, should be initiated (Figure 1).[28] Immunotherapy can be restarted once the patient improves clinically and toxicity is grade 1 or less. Appropriate HRT should be added if necessary. Relevant guidelines have recently been published by the European Society of Medical Oncology (ESMO).[28]

While the thyrotroph axis and gonadotroph function may recover, this is not common with corticotroph function. Meanwhile, low prolactin may predict lack of recovery function with a specificity of 88.9%, sensitivity of 50%, negative predictive value of 57.1%, positive predictive value of 85.7% and accuracy of 66.7%.[29]

Primary thyroid disorders

The pattern of primary thyroid disturbances includes subclinical or overt hypothyroidism, painless thyroiditis with transient thyrotoxicosis, subclinical or overt hyperthyroidism and thyroid eye disease.[8,30-33]

Abdel-Rahman et al estimated that the risk of all-grade hypothyroidism and hyperthyroidism associated with ICIs therapy is significantly elevated versus controls. Relative risks were 8.26 (95% CI: 4.67-14.62) and 5.48 (95% CI: 1.33-22.53), respectively.[21]

In a meta-analysis of PD-1 inhibitors studies the risk of hyperthyroidism was significantly increased compared to anti-CTLA4 (RR 2.45, 95% CI: 1.19-5.03), while the risk of hypothyroidism, although elevated, did not reach significance (RR: 4.13, 95% CI: 0.85-20.17).[22] The combination of nivolumab/ipilimumab increased the risk of all-grade hyperthyroidism (RR: 9.13 95% CI: 3.07-27.11) and hypothyroidism (RR: 2.01 (95% CI: 0.60-6.70), although again this was not significant for the latter.[22]

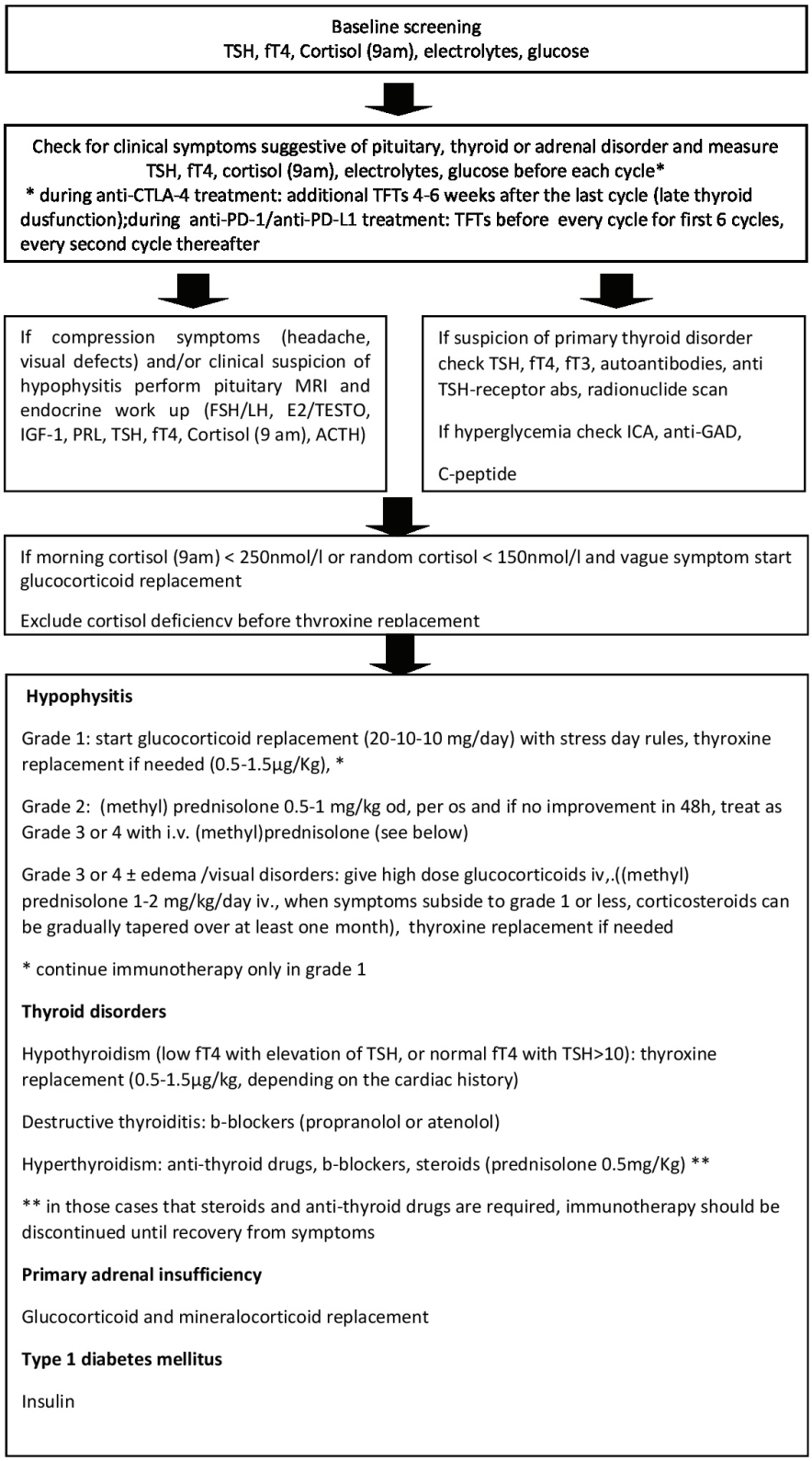

Figure 1. Proposed algorithm for the management of immune related adverse events. FSH: follicle stimulating hormone, LH: luteinizing hormone, E2: estradiol, TESTO: testosterone, IGF-1: insulin growth factor-1, PRL: prolactin, fT4: free T4, TSH: thyroid stimulating hormone.

In a series of 126 patients treated with ipilimumab for melanoma, one developed primary hypothyroidism, while eight (6%) developed subclinical hypothyroidism and 20 (16%) subclinical hyperthyroidism. Of the 46 patients who were treated with anti-PD1 inhibitors for melanoma, six (13%) developed primary hypothyroidism, a further six (13%) subclinical hypothyroidism and six (13%) developed subclinical hyperthyroidism. Of the 23 patients treated with the combination of ipilimumab and nivolumab for melanoma, four patients (22.2%) developed hypothyroidism, one of whom had a preceding episode of hyperthyroidism, and two had preceding subclinical hyperthyroidism. One patient (5.6%) had subclinical hypothyroidism and a further four patients (22.2%) had subclinical hyperthyroidism without subsequent hypothyroidism. Destructive thyroiditis was more common than Graves' disease.[31] In a study of 93 pembrolizumab treated patients 13 (14%) developed abnormal thyroid function tests. Thyroiditis occurred in seven patients (54%), four of whom recovered. New onset hypothyroidism, either overt or subclinical, developed in three patients. Thyroperoxidase (TPO) antibodies were positive only in 4/13 (31%) and diffusely increased 18-FDG uptake of the thyroid gland was observed in 7/11 (64%) patients. The authors noticed an increased number of circulating CD56+CD16+ NK cells and an elevated surface expression of HLA-DR in the inflammatory intermediate CD14+CD16+ monocytes and assumed that this may explain the destruction of the thyroid.[33]

The onset of thyroid disease usually occurs after 2-4 cycles of therapy. Aggravation of existing autoimmunity has been reported;[33-35] however, whether individual genetic susceptibility plays a role has not yet been established. Delivanis et al reported recovery of pembrolizumab-induced thyroiditis in 4/7 (57%) patients.[33] It is noteworthy that two cases with hyperthyroidism who required high-dose systemic corticosteroids for the management of other immune-related toxicities did not become hypothyroid.[31] The authors assumed that immunosuppressive therapy may prevent the development of hypothyroidism in those with ICIs-related thyroiditis. This is in contrast to the situation in ipilimumab-induced hypophysitis, where high dose steroids have not been shown to reverse pituitary dysfunction.[37]

TFTs, thyroid autoantibodies and TSH-receptor antibodies and a radionuclide thyroid uptake scan may aid in proper diagnosis.

It is suggested that in patients with symptomatic hyperthyroidism treatment with beta-blockers (propranolol or atenolol/metoprolol) should be started. Symptoms of transient hyperthyroidism due to destructive thyroiditis may be alleviated with beta blockers, as in that case antithyroid drugs are not indicated.[28] In the case of painful thyroiditis prednisolone 0.5mg/kg may be started with subsequent tapering. In the event of positive anti-TSH receptor antibodies carbimazole may be initiated. Treatment with ICIs should be interrupted until recovery from symptoms.[28]

In the case of hypothyroidism, thyroxine replacement 0.5-1.5 μg/Kg should be initiated. In subclinical hypothyroidism, substitution with thyroid hormone should be considered in the case of fatigue or other complaints that could be attributed to hypothyroidism. ICIs may be continued.[28]

A decrease in TSH levels across two measurements with normal or lowered fT4 may indicate the development of hypophysitis and thus weekly cortisol measurements should also be performed.[28]

Adrenalitis

Case reports of primary adrenal dysfunction with the use of immune checkpoint inhibitors have been published, while patients receiving these drugs require education on the potential risks of hypocortisolemia, which may be fatal if left untreated.

In the review by Byun et al, two cases of primary adrenal insufficiency are reported amongst 256 patients (0.8%) treated with ipilimumab.[20,38,39] Trainer et al described a patient with hyponatremia secondary to nivolumab-induced primary adrenal failure. The patient's FDG PET CT scan demonstrated bilateral increased FDG activity in the adrenals consistent with autoimmune adrenalitis.[40] Adrenal insufficiency of unspecified cause has been reported in patients treated with tremelimumab, nivolumab and the combination of anti-CTLA4 and anti-PD1 agents.[42] Unfortunately, most of the reports do not specify the etiology of adrenal insufficiency. Overall, the relative risk of adrenal insufficiency of any cause was significantly

elevated (RR: 3.87 (95% CI: 1.12-13.41) as described in a review by Abdelrahman et al.[21]

Although hyponatremia can occur in patients with ACTH deficiency, the possibility of primary adrenal failure should also be considered and investigated by measurement of renin, aldosterone and ACTH. There have been reports of radiological evidence of adrenalitis with normal endocrine function, following ICIs therapy, consistent with a subclinical form of adrenalitis.[39] When adrenal enlargement is observed in patients receiving immune checkpoint drugs, assessment of adrenal function through the measurement of ACTH and cortisol levels, as well as a Synacthen stimulation test, should be performed so as to rule out primary adrenal insufficiency.

Type 1 diabetes mellitus

Type 1 diabetes mellitus (T1DM) is a rare complication of anti-PD1 or anti-PDL1 agents. Antibodies against islet cell antigens have been detected in a few of the reported cases.[43,44] Time to development ranges from less than 1 month to 1 year. Insulin therapy is the mainstay of treatment. The use of glucocorticoids was not able to reverse T1DM in a patient treated with pembrolizumab for metastatic melanoma.[45]

The proposed mechanism for the development of T1DM is that lower PD-1 expressing CD4+ cells may lead to activation of autoreactive T cells that can infiltrate pancreatic islet cells and ultimately lead to T1DM.[41] It is suspected that high-risk HLA class I and II genotypes may be associated. It is recommended that clinicians managing patients undergoing anti-PD1 or anti-PDL1 therapy should carefully monitor patients for elevated levels of blood sugar, while antibodies against islet cell (ICA) and glutamic acid decarboxylase (GAD) and C-peptide should be measured in order to distinguish between T1DM and T2DM (Figure 1).

CONCLUDING REMARKS

ICIs targeting CTLA-4 and PD-1/PDL-1 have dramatically changed the outcome of patients with several advanced-stage malignancies. However, their use is associated with unique IRAEs (hypophysitis, thyroid disorders, adrenalitis, type 1 diabetes mellitus) which, though mostly transient and mild, can occasionally be fatal. At the time this manuscript was being prepared a case of primary hypoparathyroidism caused by ipilimumab and nivolumab and manifesting with acute severe symptomatic hypocalcemia was reported as a new IRAE.[46] Rapid identification of these IRAEs and appropriate treatment can improve the outcome of immunotherapy.

It is important that patients on ICIs are informed about the potential endocrine sequelae of these treatments and especially the risk of developing cortisol deficiency due to either pituitary or adrenal failure. Close monitoring and constant vigilance for the occurrence of IRAEs are necessary. As T-cell checkpoint inhibitors are now achieving regulatory approval in many types of cancer, more data on conducting the long-term follow-up of these patients is urgently required.

LEARNING POINTS

The relative risk of hypophysitis is high in those treated with anti-CTLA4 agents.

When compared with ipilimumab, the risk of all-grade hyperthyroidism and hypothyroidism with PD-1 inhibitors monotherapy seems to be higher, while the risk of all-grade hypophysitis is lower.

Patients treated with a nivolumab/ipilimumab combination therapy have a significantly increased risk of all-grade hyperthyroidism and hypophysitis when compared with ipilimumab monotherapy.

Adrenalitis and type 1 diabetes mellitus are rare IRAEs of immune checkpoint inhibitors.

Long-term follow-up of patients treated with T-cell checkpoint inhibitors is needed so that more data may be gathered on the immune-related endocrinopathies.

DECLARATION OF INTEREST

The authors declare that they have no conflict of interest.

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Review

Diabetic nephropathy: is it always there? Assumptions, weaknesses and pitfalls in the diagnosis

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ABSTRACT

Diabetic nephropathy is defined as a microvascular complication of the kidneys induced by diabetes mellitus and is characterized by albuminuria and progressive loss of kidney function. However, neither albuminuria nor glomerular filtration rate decline are diabetic nephropathy-specific markers, thus the diagnosis of diabetic nephropathy greatly depends on assumptions. Several factors should be taken into account when urinary albumin levels are assessed before establishing the diagnosis of diabetic nephropathy, while newer more specific markers for diabetic nephropathy are urgently needed.

Key words: Diabetic nephropathy, Diabetic retinopathy, Microalbuminuria, Non-proteinuric diabetic kidney disease, Renal handling of albumin, SGLT1/SGLT2, Urine proteomics

INTRODUCTION

Diabetic nephropathy (DN) is defined as the microvascular complication of the kidneys induced by diabetes mellitus and is characterized by albuminuria and progressive loss of kidney function. It is considered as one of the microvascular complications of diabetes along with retinopathy and neuropathy.[1,2] DN is vitrually the leading cause of end-stage kidney disease (ESKD).[3-5] The prevalence of DN varies enormously between continents, countries and even between regions of the same country.[5-9]

However, the diagnosis of DN is an exclusion diagnosis depending on the presence of at least microalbuminuria in a patient with a history of diabetes of at least 5 years.[10] A kidney biopsy, which is the gold standard for definitive diagnosis, treatment guidance and prognosis for other types of nephropathies, is not indicated in diabetic patients, since the risk of such an intervention is not justified. This is mainly because there are no other treatment options available at present for DN beyond the current application of optimal control of diabetes, hypertension and dyslipidemia and lifestyle modification.[10,11]

Therefore, it could be postulated that the diagnosis of DN is highly subjective, depending on the doctor's judgment and experience when at least the above two criteria are fulfilled.

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NATURAL COURSE OF DN

The natural course of DN was first described by Mogensen et al.[12] Their description was in fact based

on patients with type 1 DM, where the oncet is more or less obvious, and not on patients with type 2 DM, where the oncet is less pronounced and diagnosis may be delayed for 3-5 years.

According to Mogensen et al there are 5 stages in the course of DN (Figure1):

Stage 1, the *stage at diagnosis*, is characterized by hyperfiltration-increased estimated glomerular filtration rate (eGFR) and hypertrophy (increased kidney size). Increase in urinary albumin excretion can be present, aggravated during physical exercise, but these changes are at least partly reversible by insulin treatment.

Stage 2, the *silent stage*, develops over many years, without signs of clinical disease but still with characteristic morphologic lesions on biopsy specimens (glomerular basement membrane thickening, mesangial expansion). Estimated GFR may still be increased and albuminuria is transient. If diabetes is well controlled, albumin excretion is normal; however, physical exercise leads to an increase in albuminuria. By contrast, poor diabetes control leads to increased albumin excretion both during exercise and at rest. A number of patients continue in stage 2 throughout their lives.

Stage 3, the *incipient diabetic nephropathy stage*, is characterized by abnormally elevated urinary albumin excretion, within the microalbuminuria range 30-300mg/24h. Estimated GFR is still high or at least normal. Blood pressure is rising and albumin excretion is higher in patients with increased blood pressure.

Stage 4, the *classic overt diabetic nephropathy stage*, is characterized by persistent proteinuria (>0.5 g/24h) and persistent high blood pressure and, if left untreated, eGFR declines at a mean rate of 1 ml/min/1.73m^2/month. Long-term antihypertensive treatment reduces this eGFR decline rate by about 60% and delays uremia.

Stage 5 is *end-stage renal failure* with uremia due to diabetic nephropathy.

RETINOPATHY AND DN

Diabetic nephropathy (DN) and diabetic retinopathy (DR) are considered as interrelated diabetic vascular complications since kidneys and retina share similar

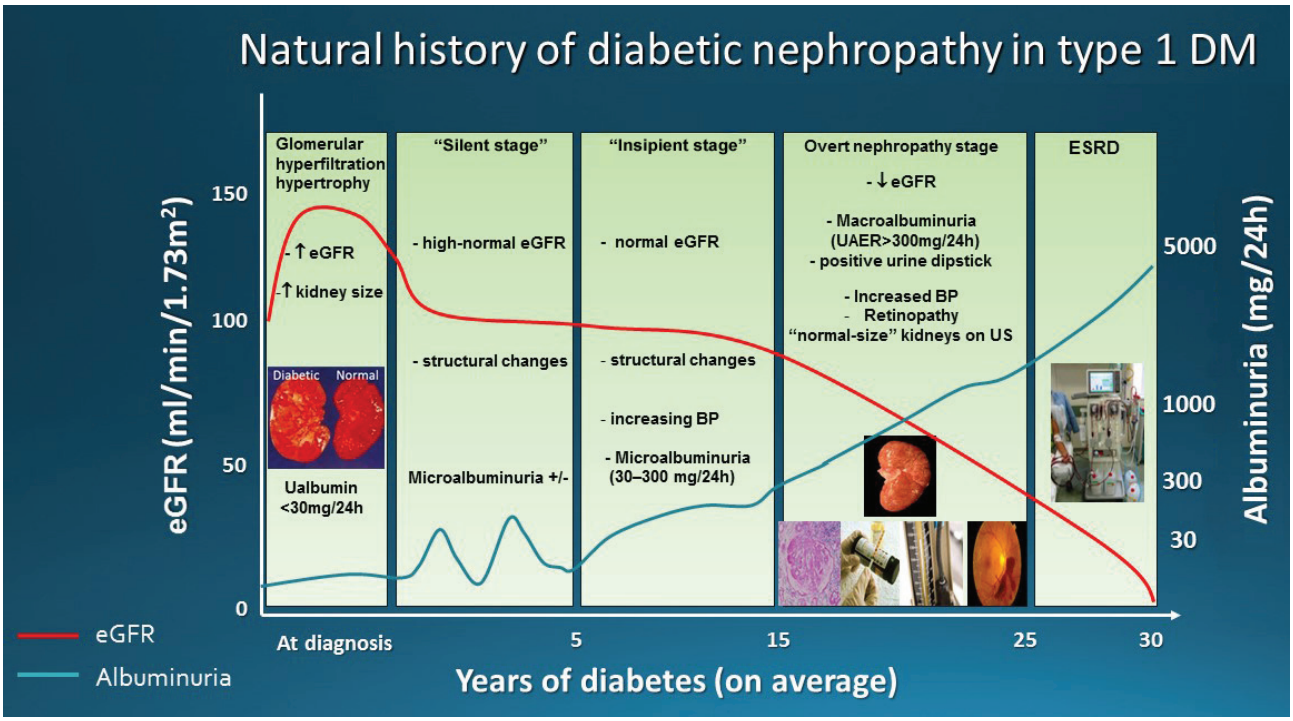

Figure 1. Natural history of diabetic nephropathy in type 1 DM, as described by Mogensen et al.

size arteries.[1,2] In clinical practice, the diagnosis of DR is used as the "*non-interventional kidney biopsy*" to diagnose DN. Therefore, in a diabetic patient with a history of DM for at least 5 years and albuminuria, with or without decrease in eGFR, the co-existence of DR strengthens the clinical suspicion that the patient also has DN. However, the absence of DR cannot exclude DN, since DR is present in approximately 60% of DN cases. Prakash et al reported an absence of diabetic retinopathy in 43% of the cases with biopsy-proven DN, while non-diabetic kidney disease was reported in 40% of the cases in the presence of DR.[13]

Diabetic retinopathy incidence varies between studies and this probably relates to the duration of the cohorts. For example, in the UK type 2 DM population studies, DR incidence was estimated to be 66% at 10 years and in the US population studies at 72.3% at 14 years.[14] Diabetic retinopathy seems to better correlate with DN in type 1 DM and much less in type 2. The prevalence of DR in type 1 DM in Europe and the USA ranges between 36.5-93.6%, while in type 2 DM in Western populations 28.5-40.3%.[14]

Pedro et al observed a prevalence of 36.47% DR in type 1 DM patients and 26.11% in type 2 DM patients.[15] In the same study, microalbuminuria was identified as a risk factor for DR in type 1 DM patients but not for type 2, whereas overt nephropathy was better correlated with DR.[15] Manaviat et al[16] showed that the prevalence of any stage of DR in the microalbuminuria stage is quite low (43%), while it increases in the overt proteinuria stage (79%), probably reflecting the more advanced diabetic vascular disease, while DR is even present in 28% of patients with normoalbuminuria.

Therefore, DR does not serve as a reliable indicator of DN in patients with type 2 DM.

MICROALBUMINURIA AS A MARKER OF ENDOTHELIAL DYSFUNCTION AND DN

Microalbuminuria (MA) can occur both in patients with DM without present or future DN as well as in patients without DM but with other types of progressive chronic kidney disease and therefore it does not serve as a specific marker for the presence of DN.[17] Mogensen et al showed that MA predicts early mortality in type 2 DM and identified MA a cardiovascular and renal risk factor in both diabetic and non-diabetic subjects.[18] Parving reported an increase in urinary albumin excretion rate in poorly controlled hypertensive patients[19] and Bigazzi et al showed that MA predicts cardiovascular events and renal insufficiency in hypertensive patients.[20] Endothelial dysfunction has been suggested as underlying the renal and/or cardiovascular organ damage observed in these diseases.[21]

The Steno hypothesis proposed that an increased permeability of the vascular endothelium constitutes a high risk for microangiopathy and a tendency to large vessel disease.[22] This systemic transvascular leakiness for albumin is associated with clinical atherosclerotic cardiovascular disease.[23] The initiating event of the atherogenesis is endothelium 'injury', e.g. by hemodynamic stress or due to dyslipidemia and, according to the 'response-to-injury' hypothesis, the increased transendothelial permeability to macromolecules is such a type of response.[24]

Therefore, while MA better predicts the development of DN in type 1 DM, MA in type 2 DM serves both as a marker of DN and of generalized endothelial dysfunction. Some type 2 DM patients with MA will not progress to the stage of overt proteinuria and these are probably the patients with hypertensive glomerulosclerosis compared to the patients that will progress and probably have diabetic glomerulosclerosis. It is obvious that in a type 2 DM patient with MA, with or without eGFR decline, but with also a long-standing history of hypertension and general atherosclerotic vascular findings, even if both of the criteria for DN are fulfilled, one could not suggest that this is DN. Longer follow-up of both eGFR and proteinuria may be needed before the diagnosis of DN is set.

REGRESSION OF MICROALBUMINURIA

Microalbuminuria may not always be a marker of an irreversible renal injury, but most likely of acute renal stress and, due to this, not rarely regression of MA is observed. In some small studies MA progression rate to overt proteinuria has been reported to be high, i.e. 85%[18] and 87%[25] risk within 6 and 14 years, respectively, although this rate may be over-

estimated. Perkins et al[26] found this rate to be at 19% in a 6-year follow-up, with approximately 60% of the patients showing regression to normal albumin excretion levels. This suggests a transient increase in albuminuria, especially in patients without optimal control of DM (HbA1c>8%) and/or of hypertension. In such patients, MA decreases or even normalizes with improvement of DM and hypertension control, but again this is not evident in all patients. Patients who do not seem to improve MA despite DM control could be individuals with more atherosclerotic disease, i.e. smokers, chronic hypertensives and patients with long-standing hyperlipidemia, where MA possibly reflects a generalized vascular endothelial disease.

Therefore, assessing MA as a marker of renal disease in type 2 diabetics should only be made after establishing good DM, blood pressure and lipids control for a reasonable time period so that the transient character of MA is excluded. According to the National Kidney Foundation recommendations for Diabetes and Chronic Kidney Disease,[27] patients with DM should be screened annually for DN. Initial screening should start 5 years after the diagnosis of type 1 DM or from a diagnosis of type 2 DM and should include measurements of urinary albumin to creatinine ratio (ACR) in a spot urine sample and serum creatinine and estimation of eGFR. Microalbumin must be measured in a first void urine sample and three samples are needed in a period of 3-6 months to establish the presence of MA.

MICROALBUMINURIA: IS WHAT IS MEASURED IN THE URINE WHAT IS LEAKING FROM THE GLOMERULUS? FACTORS INFLUENCING ALBUMINURIA LEVELS.

According to the current guidelines, for the establishment of a DN diagnosis detection of urinary albumin levels >30mg/24hours or ACR >30mg/g is crucial and mandatory. However, is the amount of albumin that we measure in the patient's urine what is really leaking from the damaged glomerulus (Figure 2)?

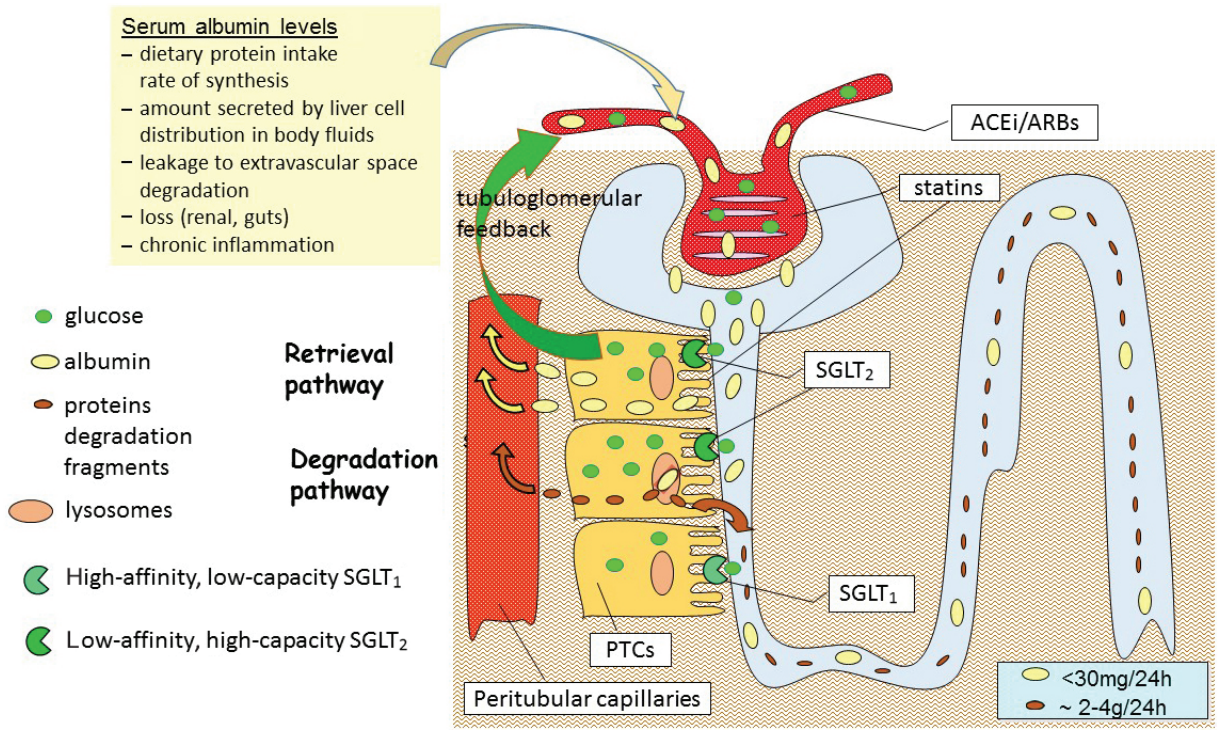

Figure 2. Factors influencing final urinary albumin levels. Albumin reabsorbed by the PTCs can return to the circulation either as intact albumin through a *retrieval transcytotic pathway* or as amino acids after being degraded in the lysosomes (*degradation pathway*). An amount of this degraded albumin is re-excreted in the urine as fractions of the initial molecule, not measured in urine samples when processed for MA measurement.

First, the amount of albumin filtered through the glomerulus depends on the serum concentration of albumin. However, not all people have the same serum albumin levels. Serum albumin levels are dependent on dietary protein intake, the rate of synthesis and the amount secreted from the liver cells, the distribution in body fluids, the level of degradation and loss.

Habitual dietary protein intake varies significantly in humans depending upon age, gender and lean body mass, well known factors that influence GFR.[28] Although there are reports suggesting that in healthy individuals dietary protein overload may not increase protein renal clearance,[29,30] animal models showed that protein overload increases proteinuria.[31,32] In humans also it was shown that consumption of excessive amounts of dietary protein promotes chronic renal disease through increased glomerular pressure and hyperfiltration[33] and might be harmful in patients with CKD.[34] Therefore, dietary differences in the amount of protein consumption may result in differences in GFR and albumin clearance and the amount of albumin measured in a urine sample.

On the other hand, hypoalbuminemia is a multifactorial process that results from a decrease in albumin synthesis as well as an increase in breakdown, leakage to the extravascular space and decreased protein intake.[35] Patients with obvious malnutrition, malabsorption syndrome (protein-losing enteropathy) or hepatic dysfunction and a chronic inflammatory state may have lower albumin serum levels due to lower intake, absorption or synthesis of albumin. Therefore, for the same degree of glomeruli damage, these patients are expected to have lower urine albumin levels due to the lower amount of plasma albumin filtered by the kidneys.

Second, for more than 30 years filtered albumin has been known to be reabsorbed by the proximal tubular cells (PTCs).[36] Despite the low, i.e. less than 30mg/day, amount of albumin found in final urine, this represents only the intact albumin that can be measured by available laboratory methods. It is known that filtered albumin is reabsorbed mainly by the proximal tubule and to a lesser extent by downstream parts of the nephron.[37] Albumin reabsorbed by the PTCs can return to the circulation either as intact albumin through a retrieval transcytotic pathway or as amino acids after being degraded in the lysosomes (degradation pathway).[38,39] An amount of this degraded albumin is re-excreted in the urine as fractions of the initial molecule, not measured in urine samples when they are processed for MA measurement (Figure 2). The protein content of the final urine has been estimated to be as high as 2 to 4 g/day[40,41] and the amount of filtered albumin has been estimated to be 50 times higher than previously assumed, suggesting that on a daily basis the normal kidney filters nephrotic levels of albumin, most of it retrieved by the proximal tubules.[42,43]

Furthermore, beyond the glomerulus, the proximal tubule also seems to contribute to DN pathology. Normally, under euglycemic conditions approximately 97% of filtered glucose is reabsorbed via the low-affinity-high-capacity Na^+-glucose cotransporter SGLT2, primarily in the early segments of the proximal tubule, and 3% is reabsorbed via the high-affinity-low-capacity SGLT1 in the late segments of the proximal tubule.[44] Hyperglycemia enhances the amounts of glucose filtered by the glomeruli and thus increases glucose delivery to both SGLT2 and SGLT1 enhancing glucose reabsorption in the proximal tubule. Glucose transporters GLUT2 and GLUT1 mediate glucose transport across the basolateral membrane, but GLUT2 may also translocate to the apical membrane in diabetes.[45]

Proximal tubular cells appear unable to decrease glucose transport rates adequately to prevent excessive changes in intracellular glucose when exposed to high glucose concentrations[46] and this leads to the notable growth phenotype of early diabetic proximal tubule hyperplasia followed by hypertrophy.[47] In the setting of normal tubuloglomerular feedback, this increased glucose tubular absorption leads to a strong tubular control of glomerular filtration in the early diabetic kidney, further enhancing glomerular hyperfiltration and proteinuria.[45,47]

Therapeutic agents, which have been developed to inhibit SGLT-2 or to effect dual inhibition of SGLT-2/SGLT-1 and are currently used for diabetes control in type 2 DM and type 1 DM, respectively, are also expected to intervene in these processes.[48,49]

SGLT2 inhibitors could potentially exert nephro-

protection not only through improved glycemic control but also through glucose-independent effects. Such effects are the blood pressure-lowering effect, since these reduce sodium reabsorption in the proximal tubule, but also afferent arteriole vasoconstriction through the tubuloglomerular feedback which leads to attenuation of diabetes-associated hyperfiltration and tubular hypertrophy and reduction in albuminuria, independent of their effects on blood pressure or glucose control.[50,51]

SGLT2 inhibition and the associated afferent vasoconstriction leads to an acute, dose-dependent reduction in eGFR by approximately 5 ml/min/1.73 m^2 and in albuminuria by approximately 30% to 40%.[52] Thus, the decrease in glomerular hyperfiltration following good diabetes control in general could in part explain the decrease in MA in clinical practice, while SGLT 2 inhibitors contribute to this through additional glucose-independent effects.

It is obvious that the anatomical and functional integrity of the proximal tubule is crucial for the final amount of albumin found in the urine and thus for the diagnosis of DN. Diseases other than diabetes that affect the PTCs,[53] but also such drugs as cisplatin, ifosfamide, tenofovir, sodium valproate and aminoglycoside antibiotics, may influence PTCs function and interfere with MA levels.[54]

Angiotensin-converting enzyme inhibitors (ACE-inh) and angiotensin II receptor antagonists (ARBs) are probably the most common classes of antihypertensive drugs prescribed in diabetic patients. Beyond their beneficial effects on cardiovascular risk factors and all-cause mortality, both categories have been shown in several studies also to have beneficial renal outcomes, including time to end-stage renal failure or doubling of creatinine, but also in preventing progression of micro- to macroalbuminuria or even remission of micro- to normoalbuminuria,[55-58] thus intervening in the result of albumin measurement in urine.

3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, referred to as "statins", widely used in diabetics for lipids control and cardiovascular protection,[59-61] have been shown also to increase albumin endocytosis by glomerular epithelial cells (podocytes), suggesting another pathway of retrieving albumin that leaks through the glomerular filtration barrier.[62-64] Conversely, statins have been shown to reduce albumin endocytosis by PTCs and may enhance albuminuria.[65,66]

Taking all the above into account, it could be postulated that the amount of albumin detected in a spot urine sample, which is the criterion in order to characterize a patient as having DN or not, is the net result of several parameters that are both patient related (degree of glomerular damage, proximal tubular integrity, serum albumin levels), but also health professionals related (use of therapeutic agents that influence glomerular filtration of albumin such as ACE-inh/ARBs or statins, or tubular processing of the filtered albumin like SGLT2 inhibitors, as well as the degree of hypertension, glycemic and lipids control in general). Therefore, for the same amount of MA detected, the degree of the actual diabetic glomerular damage may not be the same, therefore the diagnosis of DN should be individualized and carefully set after assessment of all possible parameters that may influence this process.

MICROALBUMINURIA: IS IT THE BEST WE CAN DO FOR EARLY DETECTION OF DN?

It has already been more than two decades since the first announcements of MA being an early marker of DN and, until lately, it has been regarded as the gold standard for the diagnosis and a predictor of progression to end-stage kidney disease in both type 1[67] and type 2 diabetes.[68]

However, as already analyzed above, though MA is an accessible and affordable screening marker for daily clinical practice, its predictive strength is not robust. On this account, scientists have put their efforts into identifying new markers that precede the microalbuminuria stage and are more predictive of the early stages, but also of progression of DN. In this effort, the development of new diagnostic methods and especially the omics and microRNAs technology has led to novel markers, mainly from the urine that could support this strategy.

A number of key biomarkers present in the urine that reflect the pathophysiologic processes taking place in the diabetic kidney along the nephron (glo-

merulus/podocytes, tubules) and also reflect the different mechanisms (tubular damage, oxidative stress, inflammation and activation of the intrarenal renin-angiotensin system) have been identified.[69-71] For example, podocytes injury and/or decrease in their number per glomerulus is an early finding in DN, even prior to proteinuria, and therefore podocyturia- and podocyte-specific markers in the urine, including nephrin or Wilm's tumor-1 protein, could serve as early biomarkers of DN.[72,73]

Nephrinuria was present in 100% of type 2 diabetic patients with microalbuminuria and macroalbuminuria, but also in 54% of type 2 diabetic patients with normoalbuminuria.[74] Positive urinary Wilms' Tumor-1 (WT1) protein was detected in 50% of diabetic patients without proteinuria, while in nondiabetic control subjects urinary WT1 was virtually absent.[73]

Vascular endothelial growth factor A (VEGF-A), a podocyte-derived biomarker, could also be a sensitive early marker of DN, but also a disease progression marker. VEGF urinary excretion was significantly higher in diabetics, even in the absence of albuminuria, compared to nondiabetic healthy controls, and urinary VEGF levels increased as DN advanced.[75]

Other podocytes markers could also be found early in the urine of diabetic patients, but their specificity might be an issue. For example, podocalyxin was found to increase in the urine of 53.8% of normoalbuminuric diabetic patients;[76] however, podocalyxin is also expressed in other renal and non-renal cells.[77]

Urinary transferrin may also be a sensitive marker of glomerular damage in patients with diabetes, even in the absence of albuminuria;[78] on the other hand, as with albumin it is not DN-specific, since other primary glomerular diseases also increase its excretion.[79]

Beyond podocytes (glomerular epithelium), glomerular endothelium has also been implicated in diabetic kidney disease pathophysiolgy and other microvascular complications in diabetes. Diabetic patients excrete in urine significantly more glycosaminoglycans (GAGs), part of the endothelial glycocalyx, than controls[80] and such an increase has been reported in patients with all stages of albuminuria, while urinary GACs positively correlated with disease progression.[81]

Moreover, several other proteins have been reported to increase in the urine of diabetic patients. Extracellular structural matrix proteins (collagen type IV, fibronectin, metalloproteinases), transforming growth factor (TGF)beta the potent inducer of extracellular matrix proteins, markers of tubular damage like the apical membrane receptors megalin and cubilin, the transmembrane protein of the apical membrane of PTCs-kidney injury molecule 1 (KIM-1), but also neutrophil gelatinase-associated lipocalin (NGAL) that is produced in the distal nephron are examples of such proteins. Furthermore, proteins that are normally freely filtered by the glomerulus and reabsorbed by the PCTs, including α1-microglobulin and retinol-binding protein, are also reported to increase in urine. Most of these proteins increase even in the normoalbuminuric stage,[69,70] with some of them being detected up to 5 years prior to the onset of macroalbuminuria.[71]

NON-DIABETIC NEPHROPATHY IN DIABETIC PATIENTS

When a diabetic patient develops clinical nephropathy with proteinuria, this could be due to progressive diabetic nephropathy, or another non-diabetic glomerulopathy, or both. It has been reported that another primary glomerulopathy may rarely (2-3%) implicate insulin dependent diabetes,[82] but from 10%[83] to as much as 25%[84] of non-insulin dependent diabetes cases. Among diabetic patients who clinically were suspected not to have diabetic nephropathy and underwent a renal biopsy, Soni et al[85] reported that the most common non-diabetic renal diseases were acute interstitial nephritis 18.1%, post infectious glomerulonephritis 17.24%, membranous nephropathy 11.20% and focal segmental glomerulosclerosis 7.75%.

Furthermore, even if diabetic changes such as diffuse and nodular glomerulosclerosis are found in the kidney biopsy, it is important to determine whether these are secondary changes due to diabetic nephropathy or are due to another renal disease, e.g. segmental glomerulosclerosis or idiopathic nodular glomerulosclerosis, in addition to diabetic nephropathy.[86] Other nephropathies that share similar nodular histological features with diabetic nephropathy such as membranoproliferative glomerulonephritis, mono-

clonal immunoglobulin deposition disease, amyloidosis, fibrillar glomerulopathy and idiopathic nodular glomerulosclerosis could be distinguished by detailed histopathological evaluation.[86,87]

On the other hand, many diabetic patients with chronic kidney disease may not have significant proteinuria or albuminuria even in the late CKD stages and, therefore, classic diabetic nephropathy does not appear to be the underlying renal lesion.[88] These subjects may represent almost 50% of diabetic patients with renal insufficiency and are more often older patients with a history of cardiovascular disease and usually treated with renin-angiotensin system blockers.[89,90] Disease progression in this subgroup is slower, although histological analyses may show surprisingly advanced glomerular lesions.[89] The latter histological findings are more frequently seen in type 1 DM, whereas in type 2 DM a substantial proportion of patients have more advanced tubulo-interstitial and vascular than glomerular lesions.[91,92] On this basis, Dalla Vestra et al[93] have proposed a different classification system for renal lesions in diabetic kidney disease, comprising three major groups: I) normal or near-normal renal structure (41%), II) typical diabetic nephropathology (26%) and III) atypical patterns of renal injury (33%) where there are only mild diabetic glomerular lesions and disproportionately more profound tubulo-interstitial lesions, advanced glomerular arteriolar hyalinosis and global glomerular sclerosis, in all possible combinations.

CONCLUSION

Microalbuminuria is currently used as the earliest marker for diabetic nephropathy. However, several limitations exist, since urinary albumin levels depend on several patient-related factors or health care professionals' interventions. Additionally, renal impairment could also occur even at the normoalbuminuric stage. Urinary biomarkers that are significantly elevated even in normoalbuminuric diabetic patients prior to the development of microalbuminuria could be promising biomarkers of DN at a very early stage. Nevertheless, larger multicenter prospective studies are needed to confirm their clinical utility and cost-effectiveness as a screening tool for daily practice. Until then, albuminuria must continue to be used as a marker of kidney damage in DM, but it must also be carefully assessed and monitored for a reasonable time period before setting the diagnosis of DN. If unexpectedly renal function deterioration occurs or overt proteinuria develops, nephrology consultation is advisable to exclude other primary renal pathology.

CONFLICT OF INTEREST

None.

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Review

Detection rate of somatostatin receptor PET in patients with recurrent medullary thyroid carcinoma: a systematic review and a meta-analysis

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ABSTRACT

PURPOSE: Several articles have demonstrated the high diagnostic performance of somatostatin receptor positron emission tomography (PET) in patients with neuroendocrine tumours (NETs). On the other hand, only a few studies have evaluated the detection rate (DR) of this imaging method in recurrent medullary thyroid carcinoma (MTC). We aimed to perform a systematic review and a meta-analysis of the DR of somatostatin receptor PET or PET/CT in patients with recurrent MTC to add evidence-based data to this setting. METHODS: A comprehensive computer literature search of studies published in PubMed/MEDLINE and the Cochrane Library Database through May 2017 and regarding somatostatin receptor PET or PET/CT in patients with recurrent MTC was carried out. DR was determined on a per patient-basis. A sub-analysis considering serum calcitonin (Ctn) values was also performed. RESULTS: Nine studies on the diagnostic performance of somatostatin receptor PET or PET/CT in detecting recurrent MTC were discussed in the systematic review. The meta-analysis of these selected studies provided the following DR on a per patient-based analysis: 63.5% [95% confidence interval (95%CI): 49-77]. Heterogeneity among the selected studies was found. DR of somatostatin receptor PET or PET/CT increased in patients with higher serum Ctn levels (83% for Ctn >500 ng/L). CONCLUSIONS: In patients with recurrent MTC, somatostatin receptor PET or PET/CT demonstrated a non-optimal DR which increased in patients with higher serum Ctn values. The diagnostic performance of somatostatin receptor PET or PET/CT in recurrent MTC is lower compared to that of the same imaging method in the majority of NETs.

Key words: Gallium, Medullary thyroid carcinoma, Positron emission tomography, PET/CT, Somatostatin, Thyroid cancer

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INTRODUCTION

Medullary thyroid carcinoma (MTC) is a neuroendocrine tumour (NET) originating from the neural crest-derived parafollicular C cells and accounting for approximately 1-2% of thyroid cancers. MTC may occur either sporadically or in a hereditary form as a component of the type 2 multiple endocrine neoplasia (MEN2) syndromes.[1] MTC is frequently an aggressive tumour with a high rate of metastatic spread and recurrence.[1]

The pivotal role of imaging in MTC patients is to determine the optimal treatment. The main treatment for MTC is surgical resection, which is the only strategy offering a potential cure.[1] Different morphological and functional imaging methods may be used in patients with MTC both in preoperative staging and for detecting persistent/recurrent disease after initial surgical treatment.[1]

In the post-operative setting, conventional morphological imaging modalities can often be negative or inconclusive in the presence of rising serum levels of MTC markers such as serum calcitonin (Ctn). Therefore, functional radionuclide imaging using different radiopharmaceuticals was explored as a way to detect persistent/recurrent MTC, though its role in the initial staging of MTC seems limited.[2,3] Several radiopharmaceuticals evaluating different metabolic pathways or receptor status can be used as positron emission tomography (PET) tracers in detecting MTC lesions, including fluorine-18-fluorodeoxyglucose (^{18}F-FDG), fluorine-18-dihydroxyphenylalanine (^{18}F-DOPA) and somatostatin analogues labelled with Gallium-68.[4-8]

NETs usually overexpress somatostatin receptors on their cell surface and this represents the rationale for using several somatostatin analogues labelled with the positron emitter Gallium-68 (i.e. ^{68}Ga-DOTANOC, ^{68}Ga-DOTATOC or ^{68}Ga-DOTATATE) for the diagnosis of these tumours by using PET. Somatostatin receptor PET is a valuable diagnostic tool displaying high diagnostic performance in the majority of patients with NETs, as demonstrated by several meta-analyses.[9-13] Nevertheless, experience with somatostatin receptor PET in recurrent MTC is limited compared to other NETs. Furthermore, while previous evidence-based articles have assessed the detection rate (DR) of PET using ^{18}F-FDG[14,15] and ^{18}F-DOPA[16,17] in recurrent MTC, a meta-analysis evaluating the DR of somatostatin receptor PET in recurrent MTC is still lacking. Therefore, we aimed to perform a systematic review and a meta-analysis on the DR of somatostatin receptor PET or PET/computed tomography (PET/CT) in patients with recurrent MTC to add evidence-based data to this setting.

METHODS

This meta-analysis was performed according to the "Preferred Reporting Items for Systematic Reviews and Meta-Analyses" (PRISMA) statement which describes an evidence-based minimum set of items for reporting in systematic reviews and meta-analyses.[18] Furthermore, specific suggestions for meta-analyses of diagnostic accuracy studies were followed.[19]

Search strategy

A comprehensive computer literature search of PubMed/MEDLINE and Cochrane Library Databases was conducted to find relevant published articles on the diagnostic performance of somatostatin receptor PET in evaluating patients with recurrent MTC. We used a search algorithm that was based on a combination of these terms: A) medullary OR thyroid and b) Ga OR Gallium OR DOTA* OR somatostatin and c) PET OR positron emission tomography. No beginning date limit was used. The literature search was updated until May 31st, 2017. No language restriction was applied. To expand our search, references of the retrieved articles were also screened for additional studies.

Study selection

Studies or subsets in studies investigating the diagnostic performance of somatostatin receptor PET in patients with recurrent MTC were eligible for inclusion in the qualitative analysis (systematic review). The exclusion criteria were: a) articles not within the field of interest of this review; b) review articles, editorials or letters, comments, conference proceedings; c) case reports.

For the quantitative analysis (meta-analysis) we excluded studies with insufficient data to reassess the diagnostic performance.

Two researchers (GT and AT) independently re-

viewed the titles and abstracts of the retrieved articles, applying the inclusion and exclusion criteria mentioned above. Articles were rejected if they were clearly ineligible. The same two researchers then independently reviewed the full-text version of the remaining articles to determine their eligibility for inclusion. Disagreements were resolved in a consensus meeting.

Data extraction

For each included study, information was collected concerning basic study (authors, year of publication, country of origin, study design), patient characteristics (mean age, sex ratio, number of MTC patients performing PET, sporadic or familial form of MTC), technical aspects (device used, type of radiopharmaceutical and mean injected activity, time between radiopharmaceutical injection and image acquisition, image analysis, other imaging methods performed or compared with somatostatin receptor PET such as ultrasound, computed tomography, magnetic resonance imaging, somatostatin receptor scintigraphy, metaiodobenzylguanidine scintigraphy, bone scintigraphy, pentavalent dimercaptosuccinic acid scintigraphy, ^{18}F-DOPA PET, ^{18}F-FDG PET) and applied reference standard. For each included study, the number of patients with recurrent MTC detected by somatostatin receptor PET was recorded. Patients evaluated using somatostatin receptor PET before surgery were excluded from the analysis.

Quality assessment

The 2011 Oxford Centre for Evidence-Based Medicine checklist for diagnostic studies was used for the quality assessment of the included studies. This checklist has five major parts as follows: representative spectrum of the patients, consecutive patient recruitment, ascertainment of the gold standard regardless of the index test results, independent blind comparison between the gold standard and index test results, sufficient explanation of the test to permit replication.[20]

Statistical analysis

DR of somatostatin receptor PET was calculated on a per patient-based analysis. DR was determined from the number of patients with recurrent MTC (based on increased serum Ctn values after surgery) detected by somatostatin receptor PET or PET/CT (A) and the number of patients performing somatostatin receptor PET or PET/CT (B), according to the following formula: DR = (A)/(B). Two sub-analyses considering serum Ctn values >500 ng/L and type of study (prospective *versus* retrospective) were performed. We used a random effect model for statistical pooling of the data. Pooled data were presented with 95% confidence intervals (95% CI). A I-square statistic was performed to test for heterogeneity between studies. Statistical analyses were performed using StatsDirect statistical software version 3.1 (Altrincham, UK).

RESULTS

Literature search

The comprehensive computer literature search from PubMed/MEDLINE and the Cochrane Database revealed 241 articles. Reviewing titles and abstracts, 232 articles were excluded: 192 because not in the field of interest of this review, 29 as reviews, editorials or letters, 11 as case reports. Nine articles were selected and retrieved in full-text version.[21-29] No additional studies were found screening the references of these articles. All nine articles eligible for the qualitative analysis (systematic review) were included in the quantitative analysis (meta-analysis) because all had sufficient data to assess the diagnostic performance of somatostatin receptor PET (Figure 1). The char-

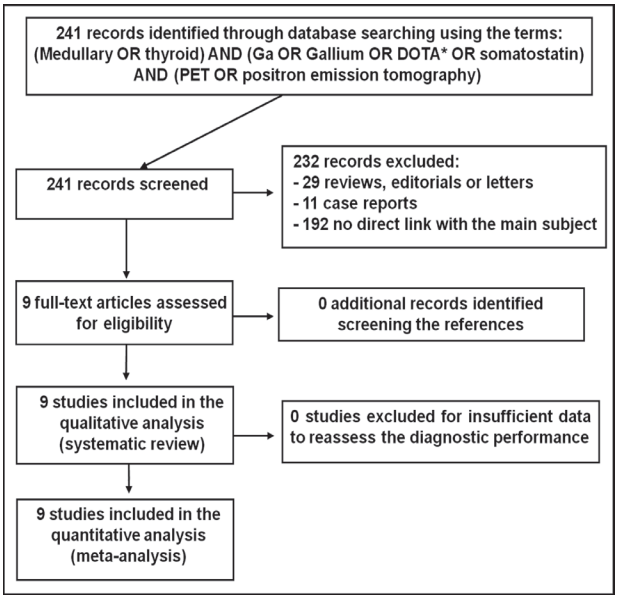

Figure 1. Flow chart of the search for eligible studies on the diagnostic performance of somatostatin receptor PET in patients with recurrent MTC.

acteristics of the nine selected studies are presented in Tables 1-4.

Qualitative analysis (systematic review)

Using the database search, nine full-text articles written over the past eight years and including 152 patients with recurrent MTC were selected.[21-29] Six articles were retrospective (67%) and three were prospective (33%). Several countries of Europe, Asia and America were represented. Concerning the sex ratio, the mean percentage of male patients in the selected studies was approximately 52.5% (Table 1).

Heterogeneous technical aspects among the included studies were found about somatostatin receptor PET. Most studies used a hybrid PET/CT device to evaluate patients with recurrent MTC; only one study used PET alone (Table 2).

Regarding the radiopharmaceuticals used, several somatostatin analogues labelled with Gallium-68 were injected (^{68}Ga-DOTATATE, ^{68}Ga-DOTANOC, ^{68}Ga-DOTANOC, ^{68}Ga-DOTALAN) in the included studies. The injected activity ranged from 72 MBq to 222 MBq, while the mean time from radiopharmaceutical injection and PET acquisition ranged from 45 to 90 minutes. The PET image analysis was performed by using qualitative (visual) analysis in all articles considering as abnormal areas of increased radiopharmaceutical uptake beyond the sites of physiological uptake and excretion. Semi-quantitative analysis based on the calculation of maximal standardized uptake values (SUVmax) of the lesions was performed in some articles. All the included articles used a combined reference standard based on pathology or imaging or clinical/biochemical/imaging follow-up data (Table 2).

The other imaging modalities performed in the included studies were quite different (Table 2). Most articles used somatostatin receptor PET or PET/CT in patients with recurrent MTC (based on increasing serum tumour marker levels) and negative conventional imaging techniques.

The diagnostic performance of somatostatin receptor PET in recurrent MTC was usually compared to that of other functional imaging techniques in this setting.

In seven articles somatostatin receptor PET findings in recurrent MTC patients were compared with those of ^{18}F-FDG PET or PET/CT with conflicting results.[22-26,28,29] Most studies did not find any significant differences in the diagnostic performance of somatostatin receptor PET/CT and ^{18}F-FDG PET or PET/CT;[23-26,29] only one study found a s ignificantly higher diagnostic performance of somatostatin receptor PET compared to ^{18}F-FDG PET/CT.[22]

Only one study compared the diagnostic performance of somatostatin receptor PET/CT with ^{18}F-DOPA PET/CT in recurrent reporting a significantly lower

Table 1. Basic study and patient characteristics

Authors	Year	Country	Study design	Patients performing somatostatin receptor PET or PET/CT	Mean age (years)	%Male	Type of MTC patients
Yamaga et al[21]	2017	Brazil	Prospective	15	43.6	46.6%	10 SP, 4 MEN2A, 1 MEN2B
Tran et al[22]	2015	UK	Retrospective	7	45	42.8%	NR
Ozkan et al[23]	2015	Turkey	Retrospective	22	42.9	50%	17 SP, 4 MEN2A, 1 MEN2B
Traub-Weidinger et al[24]	2015	Austria	Retrospective	8	NR	NR	NR
Treglia et al[25]	2012	Italy	Retrospective	18	53.1	33.3%	16 SP, 1 MEN2A, 1 MEN2B
Naswa et al[26]	2012	India	Prospective	52	44.7	73%	NR
Lapinska et al[27]	2011	Poland	Retrospective	4	NR	NR	NR
Palyga et al[28]	2010	Poland	Prospective	8	55.6	50%	NR
Conry et al[29]	2009	UK	Retrospective	18	54	72.2%	18 SP

NR: not reported; SP: Sporadic MTC; MEN: multiple endocrine neoplasia.

Table 2. Technical aspects of somatostatin receptor PET in the included studies

Authors	Device	Radiopharmaceutical	Mean radiopharmaceutical injected activity	Mean time between injection and image acquisition	PET image analysis	Imaging methods performed and compared with somatostatin receptor PET	Reference standard used
Yamaga et al[21]	PET/CT	^{68}Ga-DOTATATE	185 MBq	60 min	Visual	somatostatin receptor SPECT/CT	pathology or imaging or clinical/biochemical/imaging follow-up data
Tran et al[22]	PET/CT	^{68}Ga-DOTATATE	72 MBq	45 min	Visual and semi-quantitative	US, CT, MRI, ^{18}F-FDG PET/CT, ^{123}I-MIBG scintigraphy	pathology or imaging or clinical/biochemical/imaging follow-up data
Ozkan et al[23]	PET/CT	^{68}Ga-DOTATATE	111-148 MBq	45-60 min	Visual	^{18}F-FDG PET/CT, ^{99m}Tc (V) DMSA scintigraphy	pathology or imaging or clinical/biochemical/imaging follow-up data
Traub-Weidinger et al[24]	PET	^{68}Ga-DOTALAN, ^{68}Ga-DOTATOC	100-150 MBq	90 min	Visual	^{18}F-FDG PET	pathology or imaging or clinical/biochemical/imaging follow-up data
Treglia et al[25]	PET/CT	^{68}Ga-DOTANOC, ^{68}Ga-DOTATOC	1.5-2.5 MBq/kg	50-70 min	Visual	^{18}F-DOPA PET/CT, ^{18}F-FDG PET/CT	pathology, imaging and clinical/biochemical imaging follow-up
Naswa et al[26]	PET/CT	^{68}Ga-DOTANOC	148-222 MBq	45-60 min	Visual and semi-quantitative	^{18}F-FDG PET/CT	pathology or imaging or clinical/biochemical/imaging follow-up data
Lapinska et al[27]	PET/CT	^{68}Ga-DOTATATE	111-185 MBq	45-60 min	Visual and semi-quantitative	-	pathology or imaging or clinical/biochemical/imaging follow-up data
Palyga et al[28]	PET/CT	^{68}Ga-DOTATATE	120-185 MBq	60 min	Visual	US, MRI, CT, bone scintigraphy, ^{18}F-FDG PET	pathology, imaging and clinical/biochemical imaging follow-up
Conry et al[29]	PET/CT	^{68}Ga-DOTATATE	120-220 MBq	45-60 min	Visual and semi-quantitative	^{18}F-FDG PET/CT	pathology, imaging and clinical/biochemical imaging follow-up

PET: Positron emission tomography; CT: Computed tomography; SPECT: Single photon emission computed tomography; MRI: Magnetic resonance imaging; US: Ultrasonography; ^{18}F-FDG: Fluorine-18-fluorodeoxyglucose; ^{123}I-MIBG: Iodine-123-metaiodobenzylguanidine; ^{18}F-DOPA: Fluorine-18-dihydroxyphenylalanine; ^{99m}Tc (V)DMSA scintigraphy: Technetium-99m pentavalent dimercaptosuccinic acid.

Table 3. Detection rate of somatostatin receptor PET on a per patient-based analysis in patients with recurrent medullary thyroid carcinoma

	All Ctn values		Ctn >500 ng/L		Ctn >1000 ng/L	
Authors	**Patients true positive at PET**	**Patients performing PET**	**Patients true positive at PET**	**Patients performing PET**	**Patients true positive at PET**	**Patients performing PET**
Yamaga et al[21]	13	15	13	13	11	11
Tran et al[22]	5	6	5	5	2	2
Ozkan et al[23]	15	22	11	14	9	10
Traub-Weidinger et al[24]	6	8	NR	NR	NR	NR
Treglia et al[25]	6	18	4	9	3	7
Naswa et al[26]	42	52	NR	NR	NR	NR
Lapinska et al[27]	1	4	NR	NR	NR	NR
Palyga et al[28]	2	8	2	2	-	-
Conry et al[29]	13	18	5	5	4	4

Ctn: Calcitonin; NR: Not reported.

DR of somatostatin receptor PET/CT compared to ^{18}F-DOPA PET/CT in this setting.[25]

On the other hand, Yamaga et al. found a significantly higher number of MTC lesions detected by somatostatin receptor PET compared to somatostatin receptor scintigraphy performed with tomographic acquisition (SPECT/CT),[21] while Ozkan et al. found a significant number of MTC lesions detected by somatostatin receptor PET compared to scintigraphy with technetium-99m -labelled pentavalent dimercaptosuccinic acid (^{99m}Tc-(V)DMSA).[23]

Overall, somatostatin receptor PET or PET/CT seems not to accurately map the disease extent in patients with recurrent MTC; however, its provision of complementary information compared to other functional imaging techniques could be useful in identifying patients suitable for consideration of therapy with somatostatin analogues.[21-29]

Quantitative analysis (meta-analysis)

The DR of somatostatin receptor PET or PET/CT in patients with recurrent MTC on a per patient-analysis ranged from 25% to 86.7% with a pooled estimate of 63.5% (95%CI: 49-77%) (Figure 2).

The included studies were statistically quite heterogeneous in their estimate of DR (I^2 = 69%).

Performing a sub-analysis based on the type of study we found that the pooled DR of somatostatin receptor PET in prospective studies was slightly higher (68%; 39-91%) compared to the pooled DR of this method in retrospective studies (60.1%; 43.7-75.5%), but with significant heterogeneity in both cases (I^2 was 53% in retrospective studies and 81% in prospective studies).

The pooled DR of somatostatin receptor PET or PET/CT was also calculated in 66 patients with serum Ctn levels >500 ng/L with a pooled estimate

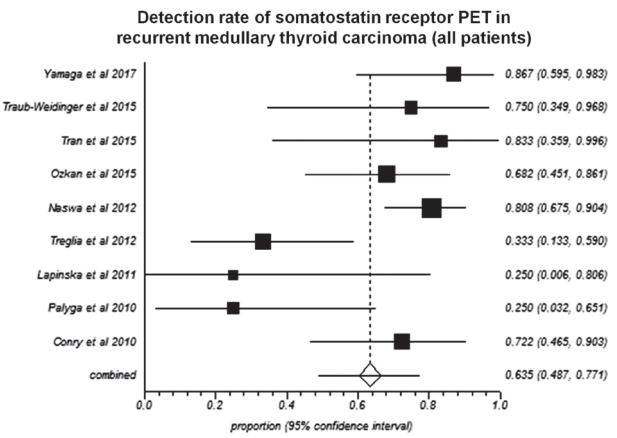

Figure 2. Plots of individual studies and pooled detection rate of somatostatin receptor PET in patients with recurrent MTC on a per patient-based analysis, including 95% confidence intervals (95%CI). The size of the squares indicates the weight of each study. Note the significant heterogeneity among the included studies.

of 83% (95%CI: 66-95%) and without significant heterogeneity (I^2 = 49%) (Figure 3).

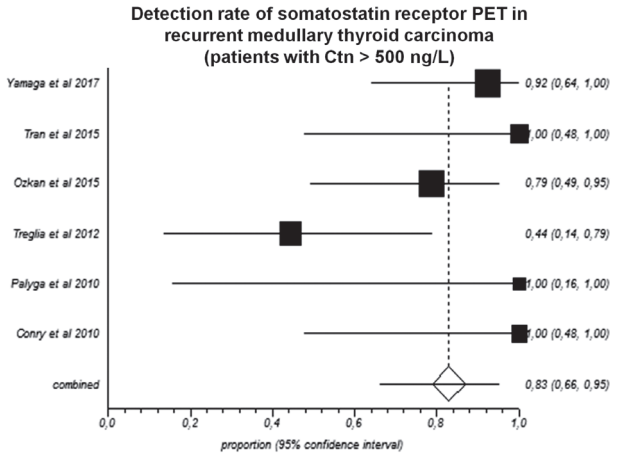

Figure 3. Plots of individual studies and pooled detection rate of somatostatin receptor PET in patients with recurrent MTC and serum calcitonin values >500 ng/L on a per patient-based analysis, including 95% confidence intervals (95%CI). The size of the squares indicates the weight of each study.

DISCUSSION

Early detection of recurrence represents a relevant step in the management of patients with MTC.[1] According to the American Thyroid Association Guidelines, if the postoperative serum Ctn level exceeds 150 ng/L MTC, patients should be evaluated by imaging procedures.[1]

To date, several studies have used somatostatin receptor PET in patients with recurrent MTC with conflicting results in terms of DR.[21-29] These studies have limited power, analyzing only relatively small numbers of MTC patients. In order to derive more robust estimates of diagnostic performance of somatostatin receptor PET in this setting we have pooled published studies. A systematic review process was adopted in ascertaining studies and the quality of the included studies was assessed by using the 2011 Oxford Centre for Evidence-Based Medicine checklist for diagnostic studies (Table 4).[20] We chose to use the DR as a measure of diagnostic performance

Table 4. Results of the quality assessment of the studies included in the meta-analysis.

First author	Spectrum of the patients	Consecutive recruitment of the patients	Ascertainment of the gold standard regardless of the index test results	Blind comparison of the index test and reference standard	Enough explanation of the index test to permit replication	2011 Oxford center for evidence based medicine level of evidence (http://www.cebm.net)
Yamaga et al[21]	Adult patients with recurrent MTC	Yes	Yes	Yes	Yes	2
Tran et al[22]	Adult patients with MTC	Yes	Yes	No	Yes	3
Ozkan et al[23]	Adult patients with recurrent or metastatic MTC	Yes	Yes	N/A	Yes	3
Traub-Weidinger et al[24]	Adult patients with MTC	Yes	Yes	N/A	Yes	3
Treglia et al[25]	Adult patients with recurrent MTC	Yes	Yes	Yes	Yes	2
Naswa et al[26]	Adult patients with recurrent MTC	Yes	Yes	Yes	Yes	2
Lapinska et al[27]	Adult patients with MTC	Yes	Yes	N/A	Yes	3
Palyga et al[28]	Adult patients with disseminated MTC	Yes	Yes	No	Yes	3
Conry et al[29]	Adult patients with recurrent MTC	Yes	Yes	No	Yes	3

N/A: not available; MTC: medullary thyroid carcinoma.

of somatostatin receptor PET and PET/CT in patients with recurrent MTC in order to homogenize the results of the various studies. In fact, the studies included in our meta-analysis were quite heterogeneous as regards the definition of false negative and true negative findings of somatostatin receptor PET: while some studies considered patients with increased serum Ctn levels and negative somatostatin receptor PET and other imaging methods as false negative results, conversely, other studies considered the same patients as true negative, thus partially contributing to the wide range of diagnostic performance that can be found in the literature. The DR overcomes these problems because both false negative and true negative results are considered in the denominator using the DR formula.

The findings of our pooled analysis indicate that somatostatin receptor PET or PET/CT has a non-optimal DR in patients with recurrent MTC on a per patient-based analysis with a pooled DR of 63.5% (slightly increasing to 68% taking into account prospective studies only). Therefore, about 40% of recurrent MTC cases would remain unidentified using this imaging method. On the other hand, it should be considered that somatostatin receptor PET is usually performed in patients with recurrent MTC after negative conventional imaging studies. Therefore, a DR of 63.5%, even if non-optimal, could affect the management of a significant number of recurrent MTC patients.

In order to study the factors which may influence the DR of somatostatin receptor PET in recurrent MTC, a sub-analysis considering serum Ctn values was performed. Our sub-analysis revealed that the DR of somatostatin receptor PET in recurrent MTC improves in patients with higher serum Ctn values (83% in patients with serum Ctn > 500 ng/L), suggesting that this functional imaging method could be useful in recurrent MTC patients with advanced disease.

Serum Ctn doubling time is a useful marker of aggressiveness and progression rate in patients with recurrent MTC. Previous meta-analyses have demonstrated that the DR of ^{18}F-FDG PET/CT and ^{18}F-DOPA PET/CT increase in patients with recurrent MTC and shorter Ctn doubling time.[14,16] Unfortunately, a sub-analysis correlating the DR of somatostatin receptor PET to Ctn doubling time in patients with recurrent MTC could not be performed due to insufficient data in the included studies. Future studies should investigate the possible relation between DR of somatostatin receptor PET and Ctn doubling time in patients with recurrent MTC.

Recent meta-analyses demonstrated a high diagnostic performance of somatostatin receptor PET or PET/CT in patients with NETs with sensitivity and specificity values higher than 90%.[10-13] Overall, the diagnostic performance of somatostatin receptor PET or PET/CT in recurrent MTC seems to be inferior compared to other NETs due to the variable somatostatin receptor expression in MTC cells.[30-34]

Different PET tracers could be used in detecting recurrent MTC.[5,35,36] Comparative analyses between ^{18}F-DOPA and ^{18}F-FDG have shown better results with ^{18}F-DOPA in terms of sensitivity and specificity and a complementary role of the two radiopharmaceuticals in the assessment of recurrent MTC.[5,25,37,38] The different behaviour of ^{18}F-DOPA and ^{18}F-FDG in recurrent MTC can be explained by their different uptake mechanisms that, in turn, reflect the different metabolic pathways of NET cells, including MTC cells. ^{18}F-DOPA is a marker of amino acid decarboxylation which is a feature of the neuroendocrine origin of MTC. It can thus be assumed that a higher ^{18}F-DOPA uptake is related to a higher degree of cell differentiation,[5,16] whereas a higher ^{18}F-FDG uptake is related to a high-proliferative activity and a poor differentiation.[5,14] Although ^{18}F-DOPA PET/CT has less prognostic value compared to ^{18}F-FDG, it can more accurately assess the extent of the disease in patients with residual/recurrent MTC,[5,38] particularly if early PET imaging is performed.[39,40]

Comparative analyses between somatostatin analogues labelled with Gallium-68 and ^{18}F-FDG have shown the complementary role of these PET tracers in recurrent MTC without statistically significant difference in terms of detection rate of MTC lesions in the majority of studies.[23-26, 29] To date, only one multicentric study compared ^{18}F-DOPA, ^{18}FDG and somatostatin analogues labelled with Gallium-68, demonstrating that ^{18}F-DOPA PET/CT is the most useful functional imaging method for detecting recurrent MTC lesions in patients with increased serum Ctn levels, performing

better than ^{18}F-FDG PET and somatostatin receptor PET and leading to a change in patient management in a significant percentage of cases.[25]

While ^{18}F-FDG and ^{18}F-DOPA may be prepared industrially (for PET centres without an on-site cyclotron) and delivered ready to use, for labelling somatostatin analogues with Gallium-68, both Germanium-68/Gallium-68 generator and lyophilised peptides are needed.[38] The easy synthesis process of radiolabelled somatostatin analogues is an advantage supporting their clinical use as PET tracers in NETs.[41]

The limited availability of ^{18}F-DOPA and somatostatin analogues labelled with Gallium-68 compared to ^{18}F-FDG is probably not a major drawback in the case of a rare cancer such as MTC, a limited number of specialised centres being able to match the demand.

Recent international guidelines for PET/CT imaging of NETs suggest the use of somatostatin receptor PET as a third option after ^{18}F-DOPA and ^{18}F-FDG PET/CT in patients with recurrent MTC.[41] Therefore, somatostatin receptor PET/CT could be useful in a minority of patients with recurrent MTC, mainly when neither ^{18}F-DOPA nor ^{18}F-FDG-PET/CT are conclusive in patients with increasing Ctn levels and negative conventional imaging methods or when treatment with somatostatin analogues is an option.[38,41] In fact, compared to ^{18}F-FDG and ^{18}F-DOPA PET, somatostatin receptor PET may have a theranostic value as this method could be useful in selecting metastatic MTC patients for therapy with cold or radiolabelled somatostatin analogues to treat metastatic lesions showing high expression of somatostatin receptors.[42]

Some limitations of our meta-analysis should be mentioned. The literature addressing the role of somatostatin receptor PET in patients with recurrent MTC is still limited (only nine studies were included in the quantitative analysis) and this could limit the statistical power of our analysis.

Heterogeneity between studies may represent a potential source of bias. The included studies were statistically heterogeneous in their estimates of DR. This heterogeneity is likely to arise through the diversity of methodological aspects of the different studies (Table 2). The baseline differences among the patients in the included studies (Table 1) and the study quality (Table 4) may also have contributed to the observed heterogeneity of the results. However, such variability was accounted for in a random effects model in our pooled analysis.[19] Furthermore, performing the sub-analysis taking into account the serum Ctn values we found a significant reduction of heterogeneity (I^2 <50%).

Publication bias is a major concern in all forms of meta-analyses, as studies reporting significant findings are more likely to be published than those reporting non-significant results. Indeed, it is not unusual for small-sized early studies to report a positive relationship that subsequent larger studies fail to replicate. We cannot exclude a publication bias in our pooled analysis.

We chose to calculate the DR on a per patient-based analysis because most of the authors have adopted this criterion. However, we cannot exclude the potential bias derived from this choice, but there were insufficient data to obtain significant results performing a per lesion-based pooled analysis. Furthermore, it was not possible to perform a sub-analysis comparing PET versus PET/CT because of insufficient data.

CONCLUSIONS

Overall our systematic review and meta-analysis showed that:

- The literature focusing on the use of somatostatin receptor PET in recurrent MTC is still limited and quite heterogeneous.
- In patients with recurrent MTC somatostatin receptor PET or PET/CT demonstrated a non-optimal DR which increased in patients with higher serum Ctn values.
- The diagnostic performance of somatostatin receptor PET or PET/CT in recurrent MTC is lower compared to that of the same imaging method in the majority of NETs.
- According to recent international guidelines, somatostatin receptor PET/CT should not be a first option for detecting recurrent MTC; however, it could be useful in a minority of patients with recurrent MTC, mainly when neither ^{18}F-DOPA nor ^{18}F-FDG-PET/CT are conclusive in patients

with increasing Ctn levels and negative conventional imaging methods or when treatment with somatostatin analogues is an option.

AUTHORS' CONTRIBUTION TO THE MANUSCRIPT

G. Treglia: literature search and review, meta-analysis, content planning and manuscript writing.

A. Tamburello: literature search and review, quality assessment, manuscript writing and language editing.

L. Giovanella: manuscript editing.

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All authors have made substantial contributions to all of the following: (1) the conception and design of the study, acquisition of data, analysis and interpretation of data, (2) drafting and revising the article, (3) final approval of the submitted version.

COMPLIANCE WITH ETHICS GUIDELINES

Conflict of interest

All authors (Giorgio Treglia, Adriana Tamburello and Luca Giovanella) declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Review

Prevalence, pathogenesis and management of prediabetes and type 2 diabetes mellitus in patients with polycystic ovary syndrome

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ABSTRACT

Polycystic ovary syndrome (PCOS) is the most common endocrinopathy in women of reproductive age. PCOS is not only the leading cause of anovulatory infertility but is also associated with an array of metabolic disorders, among which impaired glucose metabolism has been a topic of intense research. The aim of the present narrative review is to summarize the findings of the studies that have evaluated the prevalence and incidence of prediabetes and type 2 diabetes mellitus (T2DM) in patients with PCOS, to analyze the factors underpinning the association between T2DM and PCOS and to discuss the current strategies for screening and management of impaired glucose metabolism in this population. Both prediabetes and T2DM are highly prevalent in patients with PCOS. Accordingly, regular screening is recommended in this population for the early identification of impaired glucose metabolism, particularly in overweight or obese patients and in those with a family history of T2DM. Prevention of T2DM in patients with prediabetes is primarily based on lifestyle changes, while metformin might be considered in selected cases. The treatment of T2DM is similar in patients with and without PCOS but appropriate contraceptive measures should be implemented in patients receiving treatments other than insulin, metformin or glyburide.

Key words: Impaired glucose tolerance, Insulin resistance, Obesity, Polycystic ovary syndrome, Type 2 diabetes mellitus

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INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most common endocrinopathy in women of reproductive age.[1,2] PCOS is mainly characterized by oligo- or anovulation, clinical and/or biochemical hyperandrogenism and polycystic ovaries and is the leading cause of anovulatory infertility.[3,4] However, PCOS is also

associated with an array of metabolic disorders, among which impaired glucose metabolism has been a topic of intense research. Indeed, several cross-sectional and some prospective studies reported increased prevalence and incidence of impaired glucose tolerance (IGT) and type 2 diabetes mellitus (T2DM) in these patients.[5]

The aim of the present narrative review is to summarize the findings of the studies that evaluated the prevalence and incidence of prediabetes and T2DM in patients with PCOS, to discuss the factors underpinning the association between T2DM and PCOS and to present the current recommendations for screening for and management of impaired glucose metabolism in this population. A narrative review was chosen instead of a systematic review or a meta-analysis because the heterogeneity of the studies included in the review is too large, rendering the systematic review and the meta-analysis impossible to perform.

METHODS

We searched the PubMed for relevant articles using the following keywords: polycystic ovary syndrome, impaired fasting glucose, impaired glucose tolerance, type 2 diabetes mellitus, prediabetes, insulin resistance, diet, exercise, pharmacotherapy. References of retrieved articles were also evaluated for the identification of additional pertinent papers.

PREVALENCE OF PREDIABETES AND T2DM IN PATIENTS WITH PCOS

In an early case-control study in 254 patients with PCOS and 80 age- and weight-matched controls, the prevalence of IGT was 2.7 times higher in the former (31.1 vs. 14.0%, respectively).[6] Moreover, 7.5% of patients with PCOS had T2DM compared with none in the women in the control group.[6] In a more recent large study in 11,035 patients with PCOS, the prevalence of T2DM was 2.45 times higher than in age-matched controls.[7] In a meta-analysis of 13 studies that compared the prevalence of IGT between patients with PCOS and controls, IGT was 2.48 times more frequent in the former.[5] Likewise, the prevalence of T2DM was 4.5 times higher in patients with PCOS than in controls in a meta-analysis of 15 studies[5] and, importantly, these differences were similar in studies that included body mass index (BMI)-matched populations.[5] Of note, it has been estimated that 15.0-35.6% of all incident cases of T2DM in white women are attributable to PCOS.[8] Metabolic syndrome, which is associated with increased risk for T2DM,[9] is also more frequent in patients with PCOS,[10,11] while, in contrast, the prevalence of impaired fasting glucose or of HbA_{1c} levels in the prediabetic range (i.e. between 5.7 and 6.4%) appears to be low in patients with PCOS.[12,13]

INCIDENCE OF PREDIABETES AND T2DM IN PATIENTS WITH PCOS

There are very limited data on the incidence of prediabetes and T2DM in patients with PCOS. In an early uncontrolled study in 67 patients with PCOS, 9% and 8% of patients with normal glucose tolerance developed IGT and T2DM, respectively, during a follow-up period of 6.2 years.[14] Moreover, 54% of patients who had IGT at baseline developed T2DM.[14] In a more recent study in 95 patients with PCOS and age- and BMI-matched controls, the incidence of T2DM during an 8-year follow-up period was 2.3 times higher in the former (13.4% and 5.8%, respectively).[15] Moreover, obese patients with PCOS had a fivefold greater risk of developing T2DM than controls.[15] In another study, the incidence of IGT was 2.4 higher in patients with PCOS than in controls, albeit this difference did not reach significance due to the small sample size (n = 35 and 23, respectively).[16] In a more recent uncontrolled study in 255 patients with PCOS followed up for 16.9 years, the incidence of T2DM was 1.05 per 100 person-years and the age-standardized prevalence of T2DM at the end of follow-up was significantly higher than that of the general female population of a similar age (39.3 and 5.8%, respectively).[17] In another recent retrospective analysis of a large longitudinal database, patients with PCOS (n = 21,740) had a 3 times higher risk of developing T2DM during a follow-up of 4.7 years.[18] Interestingly, the incidence of T2DM was also 1.7 times higher in patients with PCOS than in BMI-matched controls.[18]

PATHOGENESIS OF PREDIABETES AND T2DM IN PCOS

Insulin resistance (IR) is intimately involved in the increased risk for prediabetes and T2DM in PCOS,[14] with IR being present in approximately 60-80% of patients with PCOS and in 95% of obese patients with this syndrome.[19,20] In addition, IR worsens with age in patients with PCOS.[21,22] The pathogenesis of IR in patients with PCOS is multifactorial.[23] Insulin-stimulated glucose uptake is mediated by the activation of phosphatidylinositol-3 kinase, while insulin-induced cell growth and differentiation is mediated through the mitogen-activated protein kinase (MAPK) http://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/mapk-erk-pathway/ extracellular signal-regulated kinase (ERK) that stimulates a cascade of enzymes, including serine/threonine, Raf, MAPK and MAPK-ERK1/2.[24] A pivotal study in obese and non-obese patients with PCOS showed that IR in this syndrome is independent of obesity and is due to impaired insulin action.[23] More recent studies also support the presence of an intrinsic IR in patients with PCOS.[25,26] Higher basal insulin secretory rates and attenuated secretory responses to meals have also been reported in patients with PCOS, which factors contribute to hyperinsulinemia in this population.[27-29] In contrast, insulin clearance does not appear to be reduced in patients with PCOS.[23]

Obesity characterizes 40-70% of patients with PCOS and is another key contributor to the pathogenesis of impaired glucose metabolism in this population.[11,30,31] In a case-control study in 254 patients with PCOS and 80 age- and weight-matched controls, both BMI and waist/hip ratio (WHR) were independent predictors of IGT,[6] while other studies have also reported higher BMI and WHR in patients with PCOS and IGT than in those with normal glucose tolerance.[32,33] In prospective studies, obesity was also independently associated with increased incidence of IGT or T2DM in patients with PCOS.[14] Obesity appears to exert a synergistic, independent, adverse effect on glucose metabolism, this accompanied by the added burden of intrinsic IR that characterizes patients with PCOS.[23] Body composition, including increased ratio of truncal/lower body fat and higher inter- and intramuscular adipose tissue also contribute to the aggravation of IR in this population.[34,35]

Hyperandrogenism might also play a role in the pathogenesis of prediabetes in patients with PCOS. Indeed, patients with PCOS and hyperandrogenemia have more pronounced IR than patients without hyperandrogenemia.[36-38] It has further been shown that treatment with androgens impairs insulin sensitivity, whereas antiandrogens improve insulin sensitivity,[39-41] while in addition it appears that IR aggravates obesity in patients with PCOS.[42,43] Moreover, several studies reported that free testosterone levels are higher in patients with PCOS and IGT than in those with normal glucose metabolism.[33]

Impaired glucose metabolism in patients with PCOS additionally appears to have a strong genetic background, since a positive family history of T2DM increases the risk for IR and prediabetes in this population.[44] Moreover, women with monozygotic twin sisters with PCOS have twice the risk of developing the syndrome.[45] In a number of studies, polymorphisms in several genes, including thyroid associated protein, DENN/MADD domain containing 1A and luteinising hormone/choriogonadotropin receptor, were associated with increased risk for PCOS.[44-48] Developmental programming, i.e. changes in gene expression due to the presence of increased steroids, mostly androgens, during fetal development, also appears to increase the risk for IR, prediabetes and T2DM in offspring of patients with PCOS.[49]

Emerging data suggest that the gut microbiome might also be implicated in the pathogenesis of impaired glucose metabolism in patients with PCOS.[50] In a recent pilot study, specific taxa of gut bacteria were associated with lower serum androgen levels and lower prevalence of oligo-amenorrhea.[51]

Muscle mitochondrial dysfunction has also been recently reported in patients with PCOS and might also play a role in the development of T2DM in this population.[52]

SCREENING FOR PREDIABETES AND T2DM IN PATIENTS WITH PCOS

According to the recent guidelines of the American Association of Clinical Endocrinologists, the American College of Endocrinology and the Androgen Excess and PCOS Society, an oral glucose

tolerance test (OGTT) should be performed every 1 to 2 years in patients with PCOS based on a family history of T2DM and a BMI >30 kg/m^2, while this test should be performed every year in patients with PCOS and IGT.[53] A recent position statement of the PCOS Special Interest Group of the European Society of Endocrinology recommends performing an OGTT in all obese patients with PCOS as well as in lean middle-aged patients (>40 years), in the presence of a personal history of gestational diabetes or family history of T2DM.[54] In contrast, both Societies mention than measurement of fasting glucose levels and HbA_{1c} appears to have limited sensitivity in identifying prediabetes in patients with PCOS.[12,13,55] The Endocrine Society recommends performing an OGTT in all patients with PCOS every 3-5 years or more frequently if central adiposity, substantial weight gain or symptoms of T2DM develop.[53] In patients unable or unwilling to perform an OGTT, measuring HbA_{1c} might be considered.[56] Moreover, the European Society of Endocrinology mentions that measurement of serum insulin and estimates of insulin resistance are not required for routine clinical management.[54]

MANAGEMENT OF PREDIABETES AND T2DM IN PATIENTS WITH PCOS

Lifestyle changes, including diet, exercise and behavior modification, represent first-line treatment for all overweight and obese patients with PCOS.[53,56,57] A hypocaloric diet (500-1000 kcal/d reduction) with reduced intake of saturated fats and increased intake of mono- and polyunsaturated fats, fiber, whole-grain breads, cereals, fruits and vegetables is recommended, along with at least 30 min of moderate-intensity physical activity daily.[56,57] A reduced carbohydrate diet also appears to result in preferential loss of abdominal fat mass.[58] It is well established that diet improves IR in patients with PCOS,[59-61] while exercise also appears to improve IR and to reduce visceral fat in this population.[62,63] Lifestyle changes reduced the risk of progression from IGT to T2DM in the general population[64,65] and in patients with PCOS.[66]

Additionally, the Androgen Excess and PCOS Society as well as the Endocrine Society suggest the use of metformin in patients with PCOS who have no improvement in IGT despite lifestyle changes and in normal weight patients with IGT.[56,57] Several studies showed that treatment with metformin improves IR and induces weight loss in patients with PCOS and that it might also induce reversion from IGT to normal glucose tolerance and prevent the development of IGT or T2DM.[67-70] However, it should be noted that in the Diabetes Prevention Program study (n = 3,234 patients with IFG or IGT), metformin was less effective than lifestyle changes in reducing the incidence of T2DM (31 and 58% reduction, respectively).[62] Importantly, the use of antiobesity agents is not recommended because of limited data on their safety and efficacy in patients with PCOS.[57,61,71]

In patients with PCOS who are diagnosed with T2DM there are no specific recommendations for the choice of antidiabetic treatment.[72] Accordingly, metformin and lifestyle changes are the treatment of choice, while any antidiabetic agent can be added in patients who do not achieve glycemic targets despite treatment with metformin (i.e. sulfonylureas, pioglitazone, dipeptidyl peptidase 4 inhibitors, glucagon-like peptide-1 receptor agonists, sodium-glucose cotransporter 2 inhibitors or basal insulin).[72] Among the available options, pioglitazone appears to improve insulin sensitivity to a similar degree as metformin, both agents exerting a synergistic effect on IR in patients with PCOS.[73,77] However, safety concerns, including the risk of weight gain and edema, limit the use of pioglitazone in this population.[54] Limited data also suggest that glucagon-like peptide 1 analogues combined with metformin attenuate IR and reduce weight more effectively than metformin monotherapy.[75,76] However, only insulin, metformin and glyburide can be safely used in pregnancy and therefore appropriate contraceptive measures should be implemented in patients receiving other antidiabetic agents.[77]

CONCLUSIONS

Both prediabetes and T2DM are highly prevalent in patients with PCOS. Accordingly, regular screening is recommended in this population for the early identification of impaired glucose metabolism, particularly in overweight or obese patients and in those with a family history of T2DM. Prevention of T2DM in patients with IGT is primarily based on lifestyle

changes, whereas metformin might be considered in selected cases. Both diet and exercise have multiple beneficial effects on glucose metabolism in this population. The treatment of T2DM is similar in patients with and without PCOS but appropriate contraceptive measures should be implemented in patients receiving treatments other than insulin, metformin or glyburide.

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Review

Differentiated thyroid cancer in childhood: a literature update

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ABSTRACT

Differentiated thyroid cancer in childhood is rare. Apart from family history, radiation exposure is a major risk factor. Although its clinical course is quite aggressive with higher rates of lymph node and pulmonary metastases as compared to adults, the final outcome tends to be favorable with mortality rates less than 2%. We herein review the clinical picture, genetic background response to treatment and recurrence rates of differentiated thyroid cancer in children and young adolescents are thoroughly reviewed and the main differences with adult differentiated thyroid cancer are highlighted.

Key words: Differentiated thyroid cancer, Mutations, Outcome, Radiation, Recurrence

EPIDEMIOLOGY

Differentiated thyroid cancer is quite rare in childhood with an annual incidence of 0.2 to 1 cases per million children. Its incidence increases after the age of 10 years and reaches a zenith in adolescence when it increases by 10-fold.[1] Specifically, the incidence rates increase from 0.43 (5-9 years) to 3.5 (10-14 years) and finally to 15.6 per million (15-19 years).[2,3] Prepubertally the rates are equal between males and females, whereas in adolescence there is a female predominence with a female to male ratio between 1-2.5 and 6, making thyroid cancer the second most common malignancy in adolescent girls[3-5] (Figure 1). In childhood, papillary thyroid cancer accounts for more than 90% of all cases[6,7] and follicular for 5-10% of cases occurring at slightly older ages.[1,5-8] Dominant histologic variants of papillary thyroid cancer in childhood are solid, follicular and diffuse

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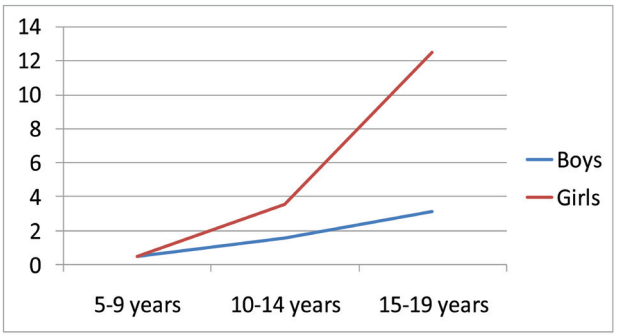

Figure 1. Incidence of thyroid cancer in childhood and adolescence.

sclerosing.[9] The incidence of NIFTP has not yet been widely investigated, although a recent study reported a rate of 1.9%, i.e. less frequent than in adults.[10] Medullary, poorly differentiated and anaplastic thyroid cancer are very rare in childhood.

RISK FACTORS

In approximately 5-10% of cases there is a family history of papillary thyroid carcinoma, comprising cases of adenomatous polyposis, DICER 1, PTEN hamartoma tumor syndrome, Carney complex or Cowden's disease.[11] However the main risk factor for PTC is radiation exposure[12,13] for children <5 years.[14,15] In particular, approximately 5 years after the 1986 Chernobyl Nuclear Power Plant accident the incidence of papillary thyroid cancer in the contaminated areas of Belarus, Ukraine and the Russian Federation increased from less than 1 case per million per year before the accident to more than 90 cases per million per year and the exposed children continued to carry an increased risk into adult life.[16] After 1996, the incidence declined progressively and after 2001 only sporadic cases, i.e. not through exposure to radiation, were reported in pediatric patients (<15 years old).[5,17-19] A non-significant increase in the prevalence of thyroid cancer was reported after the Fukushima Daiichi Nuclear Power Plant disaster where the radiation exposure was one tenth or less compared to the Chernobyl accident.[19]

MUTATIONAL STATUS

As shown in Table 1, a major difference between adult and pediatric PTC is that in children *RET/PTC* rearrangements are more common (30-70% of PTC in childhood), while activating point mutations in the signal transducing pathway, such as *RAS (NRAS* being the most frequent) and *BRAF* mutations, account for less than 10%.[20] *PET/PTC* rearrangements occur after radiation exposure or internal contamination, this being the case of the Chernobyl accident.[21] As radiation exposure induces DNA double-strand breaks, the RET gene and associated partners are highly vulnerable for recombination since they are juxtaposed in the nuclei of thyroid cells. To date, nearly 20 types of RET/PTC rearrangements have been identified.[21,22] The overall prevalence of RET/PTC diverges between sporadic and radiation-exposed pediatric PTC carcinomas (41% vs 58% respectively),[23,24] with RET/PTC3 being associated with more aggressive tumors and RET/PTC1 with classic PTC.[24,25] Due to interindividual variations in response to radiation, the role of genetic factors such as single nucleotide polymorphisms (SNPs) in the *ATM* and *FOXE1* genes in radiation-induced PTC has been documented, suggesting that its etiology may involve a DNA repair pathway, a thyroid morphogenesis pathway and/or dysregulation of the differentiated state in the thyroid.[26] In contrast to BRAF mutations, these RET/PTC rearrangements do not lead to genomic instability and dedifferentiation, which explains their better response to RAI and lower mortality rate.[7,27] Several studies have demonstrated that sodium iodine symporter expression is greater than in adults.[28,29] Moreover, a subsequent study showed that the expression of PDS, TPO and TSHR mRNA was higher in children compared to adults (22-59 years) and older patients (>60 years) as well.[30]

CLINICAL PRESENTATION

Thyroid cancer in children usually presents as a solitary nodule.[31,32] The incidence of clinically palpable thyroid nodules in children is estimated to be around 1-1.5% and in adolescents up to 13%.[33] Nodules in children carry a greater risk of malignancy compared to those in adults (22-26% versus 5-10% in most series).[34-36] It is noteworthy that even autonomously functioning hot nodules carry a higher risk of malignancy compared to those in adults, i.e. up to 30% in

Table 1. Differences of differentiated thyroid cancer in childhood compared to adults.

Nodular disease	4-fold higher risk for malignancy (22-26% vs 5-10%)
Genetic background	PET/PTC rearrangements 30-70% vs 10-20% and BRAF mutations 10% vs 40-50%
Clinical course	LMN metastases 80% vs 20-50% of adults and pulmonary metastases in 9-30% vs 2-9% of adults
Outcome	Better *NIS* expression and better response to RAI

children[37] vs 3% in adults.[38] Thyrotropin >2.5mIU/L, suspicious ultrasonographic features i.e. microcalcifications or marked hypoechogenity, irregular margins, pathologic adenopathy or multinodular goiter were identified as independent predictors of malignancy together with FNA results.[39] US characteristics and clinical context should be used for pathologic evaluation via fine-needle aspiration (FNA)[40] rather than the size criterion alone, especially in a growing child whose thyroid may be half the size of an adult's. FNA is a useful modality in the evaluation of thyroid nodules in children, with 99% accuracy and overall sensitivity and specificity of 94% and 100%, respectively.[41] According to a recent study, the risk of malignancy, including papillary microcarcinoma, is 2% for benign aspirates, 26% for AUS, 57% for FN and 100% for suspicious or malignant aspirates,[41] which is much higher than in adults.[42] In the case of indeterminate cytology, given that data regarding molecular testing in children are insufficient they cannot be used in routine clinical practice.[40] Thus, when cytology is indeterminate, surgery (lobectomy plus isthmusectomy) is favored over repeat FNA.[40] Surgery is also a reasonable option for apparently benign growing nodules or those exceeding 4 cm and causing compressive symptoms.[40]

Compared to adult papillary carcinomas, the foci are often large, multifocal and bilateral. Multifocality and capsular invasion were significantly more frequent in patients less than 16 years of age.[43] The clinical course is quite aggressive, with lymph node metastases in up to 80% of cases[44] vs 20-50% of adults[45,46] and pulmonary metastases in approximately 9-30% of cases[47] vs 2-9% of adults[48,49] (Table 1), while bone and central nervous system metastases are very rare.[50-52] Children aged <10 years have higher rates of lymph node metastases compared to older children (92.59% vs 71.43%).[53] Distant metastases occur almost always in the lungs: they are diffuse, micronodular, not detected on standard chest X-rays and usually identified by I^{131}-WBS.[51,54-57]

According to TNM classification, pediatric DTC without distant metastases is defined as stage I and in the presence of distant metastases as stage II,[40] indicating a low risk of cancer-related death. Male gender, tumor stage and lymphadenopathy are risk factors for disease free survival in stage I pediatric DTC patients.[56] According to the recent pediatric DTC guidelines, children with DTC should be categorized into three risk groups for recurrence: i) ATA pediatric low-risk patients are those with disease confined to the thyroid or with microscopic metastases to a small number of central lymph nodes (N1a), ii) ATA pediatric intermediate-risk patients are those with extensive N1a or minimal N1b disease and iii) ATA pediatric high-risk patients are those with extensive N1b and locally invasive disease (T4) with or without distant metastases.[40]

TREATMENT

The indicated surgical approach is total or near-total thyroidectomy.[40] Recurrence rates are higher with lobectomy vs total thyroidectomy.[44,49] Central neck dissection should be performed when there is evidence of central and/or lateral neck metastasis or gross extrathyroidal invasion.[40] Routine prophylactic lateral neck dissection (levels III, IV, anterior V and II) is not recommended. However, lateral neck dissection should be performed on patients with cytologic evidence of metastases to the lateral neck. Measurement of Tg in the FNA washout can be considered if the cytological diagnosis is equivocal. RAI should be administered for treatment of locoregional/nodal disease not amenable to surgery and for the treatment of distant metastases.[40] There is no benefit of RAI remnant ablation in pediatric patients with intrathyroidal disease and no lymph node disease.[58] The postoperative activity of I^{131} administration is usually 37-74 MBq/kg (1-2 mCi/kg). An alternative to the fixed doses is a pretherapeutic dosimetry; I^{131} activities that are as high as safely administrable (AHASA) are at least 200 MBq/kg but in those patients with extensive pulmonary metastases a complete dosimetry including lung dose estimate is the only method to assess safe I^{131} activity.[59] An abbreviated 2-week levothyroxine withdrawal protocol is indicated for preparation for radioiodine administration[60] due to the more rapid T4 clearance and higher TSH to free T4 ratio in children.[61] A hypothesis that may account for this difference is that kidney function is much better retained in children. Because of the expected survival time for young DTC patients, the benefits

of RAI administration should be balanced against the risks, specifically, mainly the risk of second primary malignancy which at any site corresponds to 4.4 excess cases per 10,000 person-years at risk.[62] The risk of development of a salivary malignancy is 1.7 excess cases per 10,000 person-years at risk, while the risk of developing leukemia, though slightly elevated, does not reach statistical significance.[63] The risk of pulmonary fibrosis is 1% in children with diffuse pulmonary lesions, especially when the retained I^{131} activity exceeds 80 mCi.[64] Permanent infertility does not occur in women with doses up to 300mCi I^{131} and happen in less than 10% of men with this same dose. With doses of 800mCi or more, infertility would go up to 60% of women and more than 90% of men.[65]

There is no role for external beam radiation therapy in children because usually tumor foci in the neck concentrate radioiodine and thus subsequent I^{131} therapy remission is achieved. Following thyroidectomy, levothyroxine is given at higher doses per kg of body weight compared to adults in order that TSH may be decreased to 0.1μU/ml and free T3/free T4 not exceed the above limit of normal range. The usual dose for children <10 years is 3-4μg/kg/day, while adolescents 16-18 years require 2.4-2.8μg/kg/day.[66] In children who achieve complete remission, suppressive doses are no longer mandatory and the daily levothyroxine dose may be lowered in order to maintain TSH level in the low normal range (around 0.5μU/ml).[40,67]

PROGNOSIS

Pediatric thyroid carcinoma carries an excellent prognosis. In fact, despite its aggressive presentation in childhood, often with nodular involvement and pulmonary metastases, its prognosis is more favorable than in adults,[68] showing long-term cause-specific mortality of less than 2%.[1,6,31,44,69] A systematic review demonstrated that following I^{131} treatment for pulmonary metastases complete remission is not achieved; however, disease-specific morbidity and mortality remain low (2.68%),[47] while the 10-year survival rate approaches 98%.[2] The risk of recurrence should be estimated according to the ATA risk stratification,[42] which is also valid for pediatric PTC.[70] Children <10 years in contrast to adolescents present with large[71] multifocal tumors with a solid/follicular growth pattern, extrathyroidal extension, lymph node and lung metastases and have higher recurrence rates.[70] The risk of recurrence correlates only with the extent of ETE and not with histologic subtype.[72]

Even in the case of radiation-induced thyroid cancer, an observational study of this high-risk pediatric population in the most contaminated regions near Chernobyl demonstrated that complete response [negative I^{131} whole-body scan and TSH-stimulated serum thyroglobulin (Tg) <1μg/L] was achieved in 64.2%, nearly complete remission (complete response, except stimulated Tg 1-10μg/L) in 30.1% and partial remission (Tg >10μg/L, decrease from baseline in radioiodine uptake intensity in >1 focus, in tumor volume or in Tg) in 4.8%.[73]

FOLLOW-UP STRATEGIES

Follow-up is accomplished by clinical examination, measurement of serum Tg levels, neck ultrasonography and whole body radioiodine diagnostic scans. Tg is a sensitive marker for residual or recurrent disease and should be measured along with TgAb since the presence of TgAb renders Tg results uninterpretable.[74-76] The magnitude of TSH-stimulated Tg elevation in the absence of TgAb distinguishes between patients in remission and those with recurrence. If undetectable, there is a high probability of long-term remission and the patient is mainly monitored by TSH-suppressed Tg levels, TSH is maintained in the low-normal range and the intensity of follow-up is relaxed.[40] A low-level TSH-stimulated Tg <10ng/ml indicates persistent disease requiring serial TSH-supressed measurements and radiological imaging, although this value may decline over time without additional treatment.[77] A clearly elevated TSH-stimulated Tg >10ng/ml indicates structural disease necessitating localization and intervention.[40] Cervical ultrasonography should be performed 6 months postoperatively, then annually for 5 years and thereafter for low-risk patients individualized according to the patient's risk of recurrence.[40]

In children previously treated with I^{131} a diagnostic RAI scan should be performed 12 months afterwards to confirm the absence of disease. If negative there is no need for a repeat diagnostic WBS. In contrast, for high-risk pediatric patients known to have RAI-avid metastases, a diagnostic scan is beneficial and

should be performed after at least 12 months of clinical follow-up and deferred even longer in children who continue to demonstrate a clinical response to previous treatment.[78] Low-dose diagnostic I^{123} scans are preferred over I^{131} scans due to decreased radiation exposure and avoidance of stunning.[79] However, metastatic pulmonary foci may not always be visualized through these scans.[79] Suspicious lymph nodes should be submitted to FNA and Tg measurement in the fluid aspirate. Surgery is favored for macroscopic (>1 cm) nodular disease, especially if located in a lymph node compartment not previously operated upon.[80] As previously mentioned, I^{131} is the treatment of choice for RAI-avid pulmonary metastases visualized with a diagnostic WBS.[40]

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Research paper

Surgery for pheochromocytoma: A 20-year experience of a single institution

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ABSTRACT

OBJECTIVE: Resection of pheochromocytomas is a challenging procedure due to hemodynamic lability. Our aim was to evaluate surgical outcomes in 67 patients with pheochromocytoma and to validate the role of laparoscopic surgery in the treatment of these tumors. DESIGN: This study is a retrospective review. A total of 68 procedures for pheochromocytoma were performed between June 1997 and February 2017. All patients were investigated and operated on using an established departmental protocol. Relevant data were retrieved from the hospital records of 533 patients who underwent 541 adrenalectomies for benign and malignant adrenal tumors in the same period. RESULTS: Sixty-nine tumors were removed from 67 patients. One patient with/MEN2A underwent bilateral resection of pheochromocytomas in two stages. Tumor size in laparoscopic procedures ranged from 1.2 cm to 11.0 cm (mean 5.87 cm). Thirty-seven patients had benign disease, 31 potentially malignant (based on PASS) and 1 malignant with metastasis. Eight were in the context of a familial syndrome. Forty-nine patients underwent laparoscopic adrenalectomy, 8 patients had open approach from the start for recurrent pheochromocytoma or large benign tumor, 1 patient had open approach due to inoperable malignant pheochromocytoma and 10 patients had conversions from laparoscopic to open procedure. Nine patients received sodium nitroprusside intraoperatively to treat hypertension. One patient developed pulmonary embolism and succumbed 1 month later. There were no recurrences of the benign or potentially malignant tumors during the follow-up period. CONCLUSIONS: Laparoscopic resection of pheochromocytomas, despite its increased level of difficulty compared to that of other adrenal tumors, is a safe and effective procedure.

Key words: Endocrine surgery, Laparoscopic adrenalectomy, Pheochromocytoma

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INTRODUCTION

Pheochromocytoma (PHEO) is a tumor derived from chromaffin cells of the sympathetic nervous system and its clinical symptoms are associated with

excessive production and release of catecholamines which can cause arterial hypertension and symptoms attributed to paroxysmal stimulation of the adrenergic system.[1,2]

Since the first report on laparoscopic adrenalectomy by Gagner in 1992, this approach has increasingly become the gold standard for excision of benign functioning and non-functioning tumors of the adrenal gland.[3] Laparoscopic adrenalectomy compared to traditional open resection has proven equally safe and efficient, offering a number of significant advantages such as improved access to the surgical area, less blood loss, decreased requirement for postoperative analgesia, shorter hospital stay and earlier return to normal diet and activities.[4-6]

On the other hand, in large PHEOs technical difficulties increase due to possible hemodynamic instability, tumor vascularity and malignant potential. Given the technical challenges for resection of large PHEOs, there were hesitations about using a laparoscopic approach for these tumors during the first decade of laparoscopic surgery. Meanwhile however, improvement in imaging modalities, better pharmacological preparation, advances in anaesthesia and laparoscopic surgery as well as evolving technology rendered laparoscopic surgery for PHEO safe and efficient.[7]

MATERIALS AND METHODS

A total of 68 procedures for PHEO were performed between June 1997 and February 2017. Four abdominal paragangliomas operated on during the same period are not included in this series. The preoperative diagnosis, operative details, complications, length of hospital stay, morbidity and follow-up were retrieved from the hospital records of 533 patients who underwent 541 adrenalectomies for benign and malignant adrenal tumors in the same period. Since 2008, data have been retrieved from our Department database including prospective patient records.

One patient with MEN2A syndrome underwent bilateral tumor resection with left cortex-preserving adrenalectomy.

Preoperative localization was established in all patients by computerized tomography (CT) or magnetic resonance imaging (MRI), while iodine-123 metaiodobenzylguanidine (MIBG) scan was reserved for ambiguous cases where paraganglioma or metastatic disease was suspected. All patients were investigated according to an established protocol drawn up by the Department of Endocrinology and Diabetes Center at our Hospital.

Endocrinological evaluation and complete adrenal dynamic testing were performed to determine whether the tumor was functional or not. All patients underwent basal adrenal hormonal investigation including serum cortisol, adrenocorticotropin (ACTH), dehydroepiandrosteronesulphate, renin and aldosterone levels, and the aldosterone/renin ratio.The evaluation of cortisol hypersecretion included 24-h urinary free cortisol and measurement of serum cortisol and ACTH levels after an overnight dexamethasone suppression test (1mg of dexamethasone administrated at midnight, before the morning blood sample). The evaluation of PHEOs included the measurement of urinary fractioned metanephrines (normetanephrines and metanephrines) as per local availability of diagnostic tests. Measurements of plasma free or urinary fractionated metanephrines are superior to other tests of catecholamine excess for the diagnosis of pheochromocytomas[8] and, until there are data directly comparing plasma and urinary measurements, there is no recommendation that one test is superior to the other.

In all patients, α-adrenergic blockade with a titration dose of phenoxybenzamine (starting dose 10mg - mean dose 100mg - max dose 200mg) was administered for 7-14 days prior to surgery to achieve a blood pressure of approximately 120/80mmHg. High sodium diet and intravenous fluids were administered after the second or third day of the initiation of medical treatment in order to achieve volume expansion and counteract the orthostasis associated with α-adrenergic blockade. In cases of tachyarrhythmias, a β-adrenoceptor blocker was added after the α-adrenergic blockade.

All patients had blood pressure less than 140/80mm Hg, to omit orthostatic hypotension not exceeding 80/45mmHg, no more than one ventricular extrasystole every 5 minutes and ECG without nonspecific ST segment elevations or depression as well as T wave inversions, thus ensuring adequate preoperative preparation.

SURGICAL TECHNIQUE

All the operations, open or laparoscopic, were performed with the patient in the lateral decubitus position. Therefore, there was no need to change the position of the patient in the event of a conversion to an open operation. A transperitoneal lateral approach was used to perform laparoscopic adrenalectomy.

High technology instruments and equipment are mandatory and available in the operating theater. We use a monitor tower and gas insufflator set at an intra-abdominal pressure of 14mmHg and a 30^0 10mm laparoscope. We prefer to create pneumoperitoneum with the Hasson technique to avoid any relevant morbidity. The adrenal gland is never grasped, especially in cases of PHEOs, to avoid hemodynamic instability, troublesome bleeding or tumor disruption. We ligate small vessels with clips or the harmonic scissors/scalpel. We use additional ports in obese patients or when there is a special need. Occasionally, in large tumors we use the hand-assisted technique as the last effort to avoid conversion. The specimen is placed in a special bag and extracted through minimal extension of the initial incision.

We consider early ligation of the adrenal vein to reduce the risk of hemodynamic instability. However, delayed adrenal vein ligation, following tumor mobilization, is equally safe provided that efficient pharmacological preparation is completed.

RESULTS

Sixty-nine tumors were removed from 67 patients (34 men, 33 women), with a mean age of 53.0 years (range 16-76). The mean hospital stay was 2.1 days (range 2-3 days) for the laparoscopic procedures.

The diagnosis included 37 benign PHEOs, 31 potentially malignant (based on PASS $\geq$4) and 1 malignant with metastasis. Malignancy was defined solely based on the presence of metastasis and not on a history of local recurrence, in accordance with the World Health Organization's classification of tumors.[9] One patient had von Recklinghausen disease and 2 patients had Von Hippel-Lindau syndrome. One patient with MEN2A underwent bilateral resection of PHEOs in two stages. Tumor size in laparoscopic procedures ranged from 1.2 cm to 11.0 cm (mean 5.87 cm). Operative time ranged from 55 to 210 minutes (mean 90 minutes).

Forty-eight patients underwent laparoscopic adrenalectomy, 1 patient underwent hand-assisted laparoscopic adrenalectomy, 8 patients had an open approach from the beginning due to either recurrent or large tumors (mean tumor size = 6.1 cm), 1 patient had an open biopsy due to inoperable malignant PHEO and there were 10 conversions from laparoscopic to open adrenalectomy. Six conversions were performed for hemostasis and 4 due to periadrenal tissue invasion. The relative majority of conversions (5 out of 8 laparoscopies, 62.5%) occurred in the first 5 years of this series. In the last 15 years the conversion rate dropped significantly (5 out of 51 laparoscopies,9.8%). These conversions were undertaken in order to achieve safe oncological margins and only one for hemorrhage control.

Blood transfusion was needed only in 2 patients, 1 with resection of recurrent malignancy and 1 due to preoperative anemia and minimal controlled bleeding.

Nine patients (5 laparoscopic adrenalectomies and 4 open) received sodium nitroprusside because of intraoperative hypertensive crisis (defined as a systolic blood pressure over 220mmHg sustained over 10 minutes). All significant episodes were noted during manipulation of the tumor and before ligation of the adrenal vein. Shorter episodes of hypertension that did not require pharmacological treatment with vasodilators were not included in these results. Sodium nitroprusside has a rapid onset of action but also end/termination of effect and should only be administered under close monitoring. All 9 patients had been treated with the same protocol of phenoxybenzamine titration as described above.

Transient episodes of arrhythmia or tachycardia (duration <30s) were not documented as part of the study protocol and no sustained episodes (duration >30s) of abnormal cardiac rhythm or frequency were observed. An interesting exception was a female patient with a negative preoperative work-up for PHEO and therefore no blockade, who presented hemodynamic instability during the operation and finally proved to have a potentially malignant PHEO with a PASS = 6 (PHEO of the Adrenal gland Scaled Score).

Postoperatively, there were no life-threatening complications, except for one patient who after right laparoscopic adrenalectomy developed pulmonary embolism. The patient was hospitalized in the intensive care unit and succumbed a month later.

All patients showed remarkable improvements in hypertension and reversal of the characteristic symptoms of constant adrenoreceptor stimulation by catecholamines.

No clinically significant episodes of postoperative hypoglycemia were documented.

At a mean follow-up interval of 152 months after laparoscopic adrenalectomy (range 2 months – 19.5 years), resolution of hormonal activity and no evidence of tumor recurrence were documented. The patient with the recurrent malignant PHEO survived for 2 years after the last operation. Thirty-one tumors with potentially malignant features on pathology as assessed by PASS were identified, but follow-up has not demonstrated thus far any evidence of recurrence or metastasis.

DISCUSSION

We reviewed our experience with operative treatment of pheochromocytoma to demonstrate the safety and efficacy of the laparoscopic approach and to present some noteworthy issues concerning the management of these patients. Before the advent of preoperative adrenergic blockade, morbidity and mortality associated with hemodynamic lability due to excessive catecholamine secretion were prohibitively high. Operative mortality in the pre-blockade era was reported at 13-45%, primarily due to myocardial infarction and cerebrovascular accidents.[15] Preoperative blockade has significantly reduced adverse outcomes but has not completely eliminated episodes of hemodynamic lability.[16]

Proper preparation of the patient before surgery is crucial. The preparation includes the administration of α- with or without β-blockers and intravenous expansion with crystalloids. In particular, nonselective α-adrenergic blockade (phenoxybenzamine) and selective α1-adrenergic blockade (prazocin) are used for α-blockade.[17,18] In cases of co-existing tachycardia β-blockade is employed. Intravenous volume expansion with crystalloids (2000ml/day starting on the day before surgery) is of critical importance. Continuous invasive monitoring and pharmacologic intervention by an experienced anesthetic team perioperatively are necessary to avoid substantial cardiovascular instability.[19] When the main adrenal vein is ligated, antihypertensive drugs and β-blockers administration are stopped and hemodynamics are reassessed.[20,21]

At our institution we avoid aggressive pharmacological vasodilation, especially before ligation of the adrenal vein, in order to avoid an abrupt drop in blood pressure.[16] Transient spikes in blood pressure are usually managed by limiting tumor manipulation. Guiding this practice is the fact that tumor manipulation has been shown to be the most significant intraoperative stimulus for catecholamine release during both open and laparoscopic resections.[27,30] Limiting tumor manipulation and applying pharmacological vasodilation when hypertension is persistent has resulted in no cardiovascular morbidity or mortality in our series. The sole exception was one patient who developed a severe pulmonary embolus and died after being hospitalized for a month. It should be noted that no intraoperative hemodynamic lability was observed. To our knowledge no other patient developed any form of clinically evident deep vein thrombosis. Interestingly, the incidence of clinically evident deep vein thrombosis has been reported to be as high as 7% in laparoscopic adrenalectomy.[31]

It has been suggested that the laparoscopic approach to PHEO may decrease the intraoperative release of catecholamines, compared with open surgery, thus reducing the risk of a hypertensive crisis.[32] This may be a result of fewer operative manipulations, although it seems a somewhat hyperbolic suggestion. Laparoscopy offers improved visualization and faster access to the adrenal vein further reducing the risk of catecholamine release. Other studies suggest that intra-abdominal insufflation during laparoscopy may alone cause an increase in serum catecholamines.[28,33] This is probably the result of direct tumor compression or alteration in tumor perfusion.[28]

Early ligation of the adrenal vein has historically been suggested as a prophylactic factor minimizing hormonal secretion.[34] By contrast, others endorse the safety of delayed adrenal vein ligation, challenging

the importance of the long-standing tradition of early adrenal vein ligation for these patients.[35,36] In our opinion, this technique may be even safer because the tumor is mobilized from the inferior vena cava to the right and the renal vein to the left and thus possible hemorrhage is better controlled by laparoscopy. Nonetheless, we always contemplate an initial effort to dissect the main adrenal vein at an early stage of the procedure but, if safety is jeopardized, we follow the delayed vein ligation technique.[37]

Tumor size is another consideration in laparoscopic adrenalectomy in general. In the case of PHEO, guidelines published by the Endocrine Society suggest open adrenalectomy as the treatment modality of choice in PHEOs larger than 6 cm due to increased concerns for obtaining safe oncological margins and the perceived increased risk of capsule rupture.[38] A recent limited non-randomized controlled study of large pheochromocytomas (>6 cm) comparing laparascopic adrenalectomy and open adrenalectomy indicated that recurrence rates were not statistically different between the two groups, thus questioning current guidelines.[39] However, sample sizes were relatively small and larger studies are required to fully validate laparoscopic adrenalectomy in large PHEOs. It should be highlighted that tumor size has not been conclusively proven to be a reliable predictor of recurrence or malignant potential, with different studies yielding opposing conclusions.[10,40] In our study the largest PHEO excised laparoscopically was 11.0 cm in diameter. We had 4 conversions to an open procedure because of periadrenal tumor invasion. During our follow-up there have been no instances of local recurrence. However, PHEO recurrence or metastasis can develop as long as 20 years after surgical excision and therefore all patients need to be monitored long-term.

Tumor size may also be a concern regarding conversion rates in laparoscopic surgery. However, the most common incidental cause of conversion in adrenalectomy in general is iatrogenic vascular injury.[14,41,42] As previously mentioned, PHEOs are characterized by a rich network of vessels and thus carry a higher probability of intraoperative troublesome hemorrage (Figure 1). In our series we had 6 conversions to an open procedure for successful hemostasis in patients with troublesome but not massive bleeding. It seems that laparoscopic adrenalectomy of large PHEOs is safe as long as the surgeon has the appropriate experience.[43] When using 4 cm as a cut-off value between small and large tumors there does not seem to be a significant difference in operative time and surgical outcomes.[44] In light of this, laparoscopic adrenalectomies have been performed for non-PHEO tumors up to 15 cm in diameter without any significant morbidity, therefore PHEO size does not seem to preclude a laparoscopic approach.[45-49]

Suspected malignancy remains a controversial issue.[50] Thus far, the only universally accepted criterion for malignancy in the case of PHEO is metastasis.[9] Currently there is no definitive way to predict which tumors will progress to malignancy. If there are no metastases it is impossible to precisely evaluate the degree of malignancy based on biochemical and imaging tests alone.[51]

Even pathologic evaluation is non-definitive. The most popular scale employed for estimating the risk of malignancy is the PASS, which was developed by Thompson in 2002.[51,52] Thompson proposed that tumors with a PASS score ≥4 should be considered biologically more aggressive. Nevertheless, it is has been demonstrated that this system cannot precisely

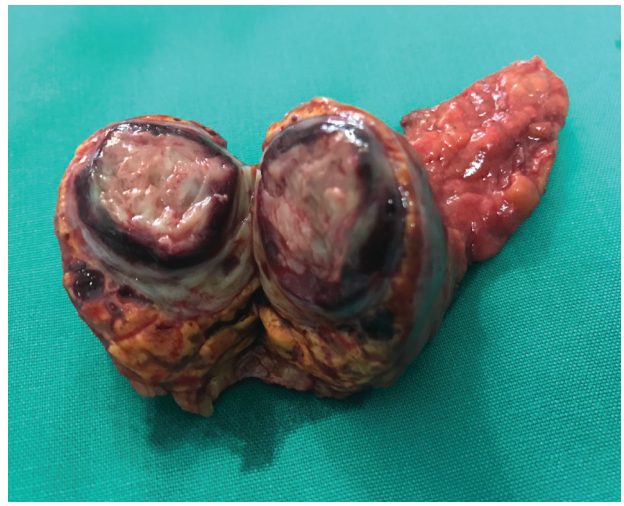

FIGURE 1. Benign adenoma of the right adrenal gland. Hemorrhagic infiltration of the adrenal cortex is evident. Preoperative imaging indicated a pheochromocytoma.

diagnose malignancy or predict the postoperative course after adrenalectomy.[51,53]

PHEOs exhibit a modest response to radiotherapy and systemic therapy (e.g. MIGB).[54] Surgical resection is currently the only therapeutic option.[55] However, there are concerns about the ability of minimally invasive techniques to totally remove the malignant lesion and avoid capsular disruption of the tumor during dissection. Laparoscopic adrenalectomy for potentially malignant (or locally aggressive) PHEOs can be performed in appropriately selected cases, in experienced centers with oncologic outcomes that are equivalent to open approaches, while providing advantages in terms of patient morbidity.[56] Proponents of minimally invasive techniques argue that in the hands of experienced surgeons, laparoscopy can be safely performed while preserving the principles of oncologic surgery, with results similar to those of open access. The basic principles during surgery are to avoid direct manipulation of or application of pressure to the tumor in order to avoid rupture of the tumor capsule.[51,57] The adrenal gland is resected en bloc with the surrounding adipose tissue and it is always extracted after being placed in a specimen retrieval bag.[51]

PHEO has been proposed as an independent factor predicting open conversion.[14] In our series our conversion rate reached 17.2%, higher than that of the laparoscopic approach for other benign adrenal lesions (8.06%).[61] However, after the eighth laparoscopic attempt conversion rates dropped significantly to 9.8% (from 62.2%), indicating that familiarity with the intricacies of this procedure have a profound effect on successful completion by laparoscopy and might indicate different learning curves compared to other adrenal tumors. When taking into account the 9.8% conversation rate, no statistical significance is noted compared to other benign adrenal lesions. The early conversion rates may be partially explained by the low threshold to convert to an open procedure during the first attempts rather than struggle to complete a laparoscopic operation by risking disruption of the tumor capsule.

In conclusion, laparoscopic adrenal resection represents a safe and effective option for the management of PHEOs. Patient selection, close preoperative and intraoperative management and adequate postoperative follow-up are essential to prevent surgery-induced uncontrollable catecholamine secretion and cardiovascular instability. Laparoscopic resection of large PHEOs necessitates experience in open and advanced laparoscopic surgery. Potentially malignant tumors should not be accounted as an absolute contraindication for laparoscopic excision but oncological principles should be strictly followed. Special effort should be made not to damage the capsule of the tumor. Nonetheless, large potentially malignant or malignant tumors >10 cm, or those with possible organ infiltration must be approached with the open technique from the beginning.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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Research paper

Baseline glucose homeostasis predicts the new onset of diabetes during statin therapy: A retrospective study in real life

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ABSTRACT

OBJECTIVE. We evaluated the risk of altered glucose levels and new-onset diabetes (NOD) associated with statins according to glucose levels at baseline in a population treated for dyslipidemia on primary prevention for >5 years. DESIGN. The retrospective study included 308 subjects (265 on statins and 43 controls on diet) with a follow-up of 5-15 years. The cohort was classified according to glucose tolerance at both baseline and follow-up. RESULTS. The cumulative incidence of NOD was 13.6% (9.3% in controls and 13.5% in treated patients). NOD was diagnosed after 3.4±1.8 years. In the group with normal glucose levels at baseline, a family history of diabetes (OR: 3.4, 95% CI 1.3-8.9), BMI >30 kg/m^2 (OR: 8.5, 95% CI 2.0-35.8), treatment with thiazide (OR: 21.9, 95% CI 1.2-384.2) and no alcohol consumption (OR: 0.3, 95% CI 0.1-0.8) reduced the risk of developing altered glucose levels or NOD. No effects of statins were seen. In the group with altered glucose levels at baseline, hypertension (OR: 5.0, 95% CI 1.0-25.3) and hypertriglyceridemia (OR: 3.5, 95% CI 1.0-11.8) increased the risk of remaining with altered glucose levels or developing NOD. Treatment with statins (OR: 7.5, 95% CI 1.5-37.4), in particular atorvastatin, was associated with an increased risk. In the whole population, statin therapy (OR: 4.0, 95% CI 1.1-14.1, p<0.020), and in particular simvastatin and atorvastatin, was associated with increased risk of altered glucose levels or NOD. Patients who developed or maintained altered glucose levels or NOD had a poor metabolic phenotype at baseline. CONCLUSIONS. Statins were associated with an increased risk of NOD or altered glucose levels, mainly in subjects with altered glucose levels before the beginning of therapy. Poor metabolic phenotype and unhealthy behaviors or family history of diabetes contributed to that risk.

Key words: Altered glucose levels, Dyslipidemia, New-onset diabetes, Statin

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INTRODUCTION

Due to the high morbidity and mortality related to cardiovascular (CV) diseases, prevention is a priority. In Europe, around 50% of deaths are caused by or are related to these pathologies (mainly coronary disease, responsible for 20% of deaths, followed by stroke).[1]

Statins represent one of the most important breakthroughs in the treatment of patients with high CV risk, as documented by many studies on both primary and secondary prevention.[2,3] However, an association has been identified between statin therapy and new onset of type 2 diabetes mellitus (NOD),[4-8] which is a major CV risk factor.[9]

In February 2012, the US Food Drug Administration (FDA) issued a warning related to statin therapy and an increased risk of NOD.[10] Nevertheless, it is useful to remember that most of the evidence has emerged from *post-hoc* analyses of studies not specifically designed to investigate NOD.

The aim of the present study was to evaluate the long-term effect of statin therapy on glucose metabolism in a heterogeneous group of dyslipidemic primary prevention patients referred to a tertiary center. The primary objective was the evaluation of NOD in patients treated with statin therapy for at least 5 years. The secondary objective was the detection of alterations in markers of prediabetes during statin treatment on the basis of anthropometric and biochemical parameters, concomitant treatment, family history, unhealthy behaviors and risk factors as described in the Treating to New Target (TNT), Incremental Decrease in End Points Through Aggressive Lipid Lowering Trial (IDEAL) and the Stroke Prevention by Aggressive Reduction in Cholesterol Levels (SPARCL) studies.[7,11-14]

SUBJECTS AND METHODOLOGY

We performed a retrospective longitudinal study from 2000 to 2014 in patients referred to the Lipidology outpatient clinic of the "Ospedale Maggiore della Carità", Novara, Italy. Study quality was assessed using the STROBE checklist.[15] Patients were selected based on the following criteria: males and females aged 18-90 years, naïve to statin use before the baseline sample followed by continuous statin (cases) or diet therapy (controls) for at least 5 years. We selected patients on primary prevention; those with a known diagnosis of type 2 diabetes and/or cardiovascular diseases different from hypertension, dyslipidemia secondary to kidney failure or cystic fibrosis, family history of type 1 diabetes or active neoplasia were excluded. Patients with impaired fasting glucose or impaired glucose tolerance at baseline were accepted. Changes of statin dosage or molecule were allowed and patients were scheduled as treated with the last dosage or molecule at the end of the study. To be included in the study, patients and controls had be checked and scheduled at baseline and at the last visit for family history (type 2 diabetes and dyslipidemia), lifestyle habits (smoke and alcohol consumption), other diseases or treatments. They also had to have evaluations of the following clinical and biochemical parameters: weight, height, waist circumference, systolic blood pressure (SBP), diastolic blood pressure (DBP), fasting glucose, HbA_{1c}, total cholesterol (T-c), High Density Lipoprotein-cholesterol (HDL-c), Low Density Lipoprotein-cholesterol (LDL-c), triglycerides (TG), serum creatinine, aspartate aminotransferase (AST) and alanine aminotransferase levels (ALT).

The initial cohort was composed of 1,879 subjects, of whom 1,571 were excluded because they did not fulfill the inclusion criteria. A total of 308 subjects were included in the study. The cohort was classified according to glucose tolerance at both baseline and follow-up. They were firstly divided into 2 groups, i.e. subjects with normal (A) and altered glucose levels (B). The subjects were further divided into patients who maintained (AA) or patients whose levels returned to normal (BA), patients who developed (AB) or who maintained altered glucose levels (BB) and patients who developed NOD (AC, BC).

Height was measured by the Harpenden stadiometer to the nearest mm and weight by using an electronic scale, both taken in triplicate. BMI was calculated as body weight divided by squared height (kg/m^2). SBP and DBP were measured using a mercury free sphygmomanometer (UM-102A, A&D Medical, Japan) with an appropriate cuff size after participants had been seated quietly for at least 15 min with their right arm supported at the level of the heart and feet flat on the floor, prior to other physical evaluations.

NOD was diagnosed according to the American Diabetes Association (ADA) criteria for fasting plasma glucose (≥126 mg/dl, 7.0 mmol/l), post-glucose challenge (2-h plasma glucose ≥200 mg/dl, 11.1 mmol/l) during an oral glucose tolerance test (OGTT) or HbA_{1c} levels ≥6.5%.

Altered glucose levels were used for individuals with impaired fasting glucose (IFG; fasting plasma glucose 100 mg/dL or 5.6 mmol/L to 125 mg/dL, 6.9 mmol/l) and/or impaired glucose tolerance (IGT; 2-h PG in the 75g OGTT 140 mg/dl/7.8 mmol/l to 199 mg/dl/11.0 mmol/l) according to the ADA criteria.[16] LDL-c was determined either by a laboratory test or with the Friedewald formula; non-HDL-c, LDL- to HDL-c ratio and T-c to HDL-c ratio were also calculated. Prediction risk factors for NOD were evaluated according to the TNT, SPARCL and IDEAL studies[7,11-14] as fasting glucose >100 mg/dl (5.6 mmol/l), blood pressure levels >140/90 mmHg or treatments for hypertension, BMI >30 kg/m^2 and TG >150 mg/dl.

Biochemical analyses were performed using standardized methods in the Hospital's Laboratory. Plasma glucose levels (mg/dl; 1 mg/dl: 0.05551 mmol/l) were measured by the gluco-oxidase colorimetric method (GLUCOFIX, by Menarini Diagnostici, Florence, Italy). HbA_{1c} levels were measured by high-performance liquid chromatography (HPLC) using a Variant machine (Biorad, Hercules, CA); intra- and inter-assay coefficients of variation are respectively lower than 0.6 and 1.6%. Linearity is excellent from 3.2% (11 mmol/mol) to 18.3% (177 mmol/mol). Serum creatinine levels were assessed using the enzymatic method of creatinine deamidase/GLDH (Adivia Chemistry - Bayer). Plasma T-c (mg/dl; 1 mg/dl: 0.0259 mmol/l) concentration was measured by esterase and oxidase conversion (Advia 1650, Bayer Diagnostics, Newbury, UK); coefficient of variation 1.9 %. Plasma TG (mg/dl; 1 mg/dl: 0.0113 mmol/l) and HDL-c (mg/dl; 1 mg/dl: 0.0259 mmol/l) concentrations were measured by enzymatic determination (Advia 1650, Bayer Diagnostics, Newbury, UK); coefficient of variation 1.7%. AST and ALT levels were measured by enzymatic methods (Roche Diagnostics, Mannheim, Germany).

The study was conducted in accordance with the declaration of Helsinki. It was approved by the Local Inter-Hospital Ethic Committee (Maggiore Hospital Ethical Committee) and written informed consent was obtained from all subjects.

Statistical analysis

Results are expressed as mean and standard deviation (SD) or 95% confidence interval (95% CI). Distributions of continuous variables were examined for skewness and were logarithmically transformed as appropriate. Analysis of variance was used to determine differences between groups. Logistic regression analysis was used to determine the association of altered glucose levels/NOD with the odds ratio (OR, 95% CI) of the following risk factors: diet/treatment with statin, alcohol, thiazide therapy and risk factors derived from TNT, IDEAL and SPRCL studies (model 1). Model 2 also included the family history of type 2 diabetes or dyslipidemia. All the models were corrected for age, sex, PUFA-n3 or treatment with ezetimibe, smoking habits and years of follow-up. Statins were also classified according to molecules (fluvastatin + pravastatin; simvastatin, atorvastatin, rosuvastatin). Logistic regression analysis was performed in the whole population or in the two subgroups (controls on diet/patients on statins) to refine the risk. Diet/statin therapy was evaluated in both main effect and custom models (interaction of all fixed factors and covariates).

Cox regression models were fitted to the time of observation to the outcome of NOD. The ascertainment of the year of diagnosis of NOD was derived from the files of all patients who had obtained an exemption from payment for drugs, syringes and glucose monitoring strips because of a diagnosis of diabetes mellitus. Cox regression models were not performed for the outcome NOD + altered glucose levels because the year of diagnosis of altered glucose levels was uncertain.

Statistical significance was defined as $p < 0.05$ (2-sided models). The statistical analysis was performed with SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

The first dataset included 1,879 subjects of whom 1,571 patients were excluded because they did not satisfy the inclusion criteria: 1,206 subjects for a

follow-up <5 years, 26 for incomplete clinical or biochemical data, 20 for statin withdrawal before 5 years and 319 for the presence of type 2 diabetes at baseline. The final dataset of 308 subjects (130 males, 178 females, age: 53.9±12.6 years), with 265 patients on statin therapy and 43 on diet (controls), included 216 patients in group A (normal glucose levels) and 92 of group B (altered glucose levels). Moreover, 123 patients had a family history of type 2 diabetes and 164 of dyslipidemia.

With respect to therapy, 65 patients had been treated with atorvastatin, 85 with simvastatin, 62 with rosuvastatin, 37 with pravastatin and 16 with fluvastatin, respectively. Additionally, 16 patients had been further treated with ezetimibe, 25 patients and 6 controls with PUFA-n3, 9 patients had been taking thiazide diuretics and 2 patients beta-blockers for hypertension. No patients had been on chronic corticosteroid treatments.

Clinical and metabolic characteristics of patients with normal and altered glucose levels at baseline

Age, weight, BMI, waist circumference, SBP, DBP, fasting glucose, HbA_{1c} and AST levels were higher ($p<0.020$), while T-c, LDL-c, HDL-c and non-HDL-c ($p<0.030$) were lower in group B than A, respectively, in the cohort as a whole or divided according to cases and controls (Table 1).

Altered glucose levels at baseline were associated with family history of type 2 diabetes (OR: 2.81, 95% CI 1.58-4.98, $p<0.001$) and dyslipidemia (OR: 0.3, 95% CI 0.168-0.536; $p<0.001$). No associations with smoking or alcohol consumption were noted.

Clinical and metabolic characteristics of patients with normal and altered glucose levels at follow-up

At the last follow-up visit, those in group B had higher body weight, BMI, waist circumference, glucose, HbA_{1c}, blood pressure, TG, ALT, while they had lower T-c, LDL-c, HDL-c, and T-c to HDL-c ratio levels than those in group A (Table 2).

By considering subjects with normal glucose levels at follow-up (AA and BA), those treated with statins had higher BMI (24.6±3.7 vs 23.4±5.0 Kg/m^2, $p=0.050$) but lower T-c (199.6±43.1 vs 227.3±48.1 mg/dl, $p<0.001$), LDL-c (114.1±41.3 vs 139.0±38.4 mg/dl $p<0.001$), non-HDL-c (129.1±55.6 vs 164.2±44.2 mg/dl, $p<0.001$), LDL-c to HDL-c ratio (2.0±1.1 vs 2.4±1.2, $p<0.048$), T-c to HDL-c ratio (1.9±0.2 vs 4.0±1.5; $p<0.047$) than controls.

By considering subjects with altered glucose levels at follow-up (AB, AC, BB and BC), those treated with statins had lower T-c (190.9±35.5 vs 211.8±38.4 mg/dl, $p<0.020$), LDL-c (108.1±32.1 vs 128.4±39.0 mg/dl $p<0.022$), non-HDL-c (133.7±37.0 vs 161.4±35.8 mg/dl, $p<0.004$), LDL-c to HDL-c ratio (2.0±0.7 vs 2.6±0.8, $p<0.006$), T-c to HDL-c ratio (1.9±0.2 vs 3.8±1.3; $p<0.004$) and AST levels (22.6±8.3 vs 25.9±7.0 U/L, $p<0.009$) than controls.

Table 1. Clinical and metabolic characteristics of patients with normal and altered glucose levels at baseline

Variable	Group A	Group B	p
Subjects (M/F)	216 (88/128)	92 (42/50)	0.760
Age (yrs)	54.0 ± 12.6	57.2 ± 11.1	0.004
Weight (Kg)	67.7 ± 12.2	75.2 ± 17.0	0.0001
BMI (Kg/m2)	24.6 ± 3.5	27.6 ± 6.2	0.0001
WC (cm)	83.4 ± 12.1	97.1 ± 12.1	0.0001
SBP (mmHg)	128.1± 16.3	134.8 ± 16.8	0.0001
DBP (mmHg)	78.6 ± 8.3	80.8 ± 7.8	0.016
FPG (mg/dl)	87.1 ± 8.0	111.0 ± 8.6	0.0001
HbA_{1c} (%)	5.5 ± 0.3	6.0 ± 0.5	0.0001
T-c (mg/dl)	273.9 ± 55.8	255.8 ± 53.3	0.006
HDL-c (mg/dl)	59.8 ± 17.2	54.7 ± 17.9	0.013
LDL-c (mg/dl)	183.3 ± 55.1	167.7 ± 53.1	0.014
Non HDL-c (mg/dl)	215.2 ± 54.1	200.6 ± 58.4	0.023
LDL-c/HDL-c	3.23 ± 1.16	3.23 ± 1.46	0.654
T-c/HDL-c	4.87 ± 1.41	4.99 ± 1.96	0.792
TG (mg/dl)	165.1 ± 138.8	176.3 ± 105.5	0.258
Creatinine (mg/dl)	1.0 ± 0.2	1.0 ± 0.2	0.243
eGFR (ml/min)	75.4 ± 15.9	70.0 ± 14.4	0.520
AST (U/L)	23.1 ± 8.2	24.0 ± 6.3	0.191
ALT (U/L)	24.3 ± 12.5	28.5 ± 10.8	0.002

ALT: alanine aminotransferase; AST: aspartate aminotransferase; BMI: Body Mass Index; DBP: Diastolic Blood Pressure; eGFR: estimated Glomerular Filtration Rate; FPG: Fasting Plasma Glucose; HbA1c: Haemoglobin A1c; HDL-c: HDL cholesterol; SBP: Systolic Blood Pressure; T-c: total cholesterol, Group A: patients with normal glucose levels; TG: triglycerides. Group B: patients with altered glucose levels. Data are expressed as mean ±SD.

Table 2. Clinical and metabolic characteristics of patients with normal (Group A) and altered glucose levels (Group B) at the follow-up

Variable	Group A	Group B	p
Subjects (M/F)	187 (71/116)	121 (59/62)	
Weight (Kg)	67.3 ± 13.7	74.8 ± 14.7	<00001
BMI (Kg/m2)	24.4 ± 3.9	27.6 ± 4.8	<0.001
WC (cm)	90.7 ± 13.5	100.3 ± 11.3	<0.001
SBP (mmHg)	124.2 ± 18.4	133.3 ± 16.2	<0.001
DBP (mmHg)	78.0 ± 7.2	80.1 ± 8.2	0.010
FPG (mg/dl)	89.4 ± 7.7	116.6 ± 15.5	<0.001
HbA1c (%)	5.5 ± 0.2	6.3 ± 0.5	<0.001
T-c (mg/dl)	204.0 ± 45.0	193.5 ± 36.4	0.010
HDL-c (mg/dl)	62.3 ± 21.0	54.2 ± 14.5	<0.001
LDL-c (mg/dl)	118.1 ± 41.8	110.7 ± 33.6	0.040
Non HDL-c (mg/dl)	134.4 ± 55.4	137.1 ± 37.8	0.366
LDL-c/HDL-c	2.1 ± 1.1	2.1 ± 0.7	0.051
T-c/HDL-c	3.5±1.3	3.7 ± 0.9	0.004
TG (mg/dl)	129.1 ± 87.7	147.7 ± 67.3	0.001
Creatinine (mg/dl)	0.8± 0.2	0.8 ± 0.1	0.351
eGFR (ml/min)	86.9 ± 16.8	84.9 ± 17.5	0.141
AST (U/L)	21.9± 6.8	23.1 ± 8.2	0.163
ALT (U/L)	22.6 ± 11.2	31.2 ± 18.2	0.003

ALT: alanine aminotransferase; AST: aspartate aminotransferase; BMI: Body Mass Index; DBP: Diastolic Blood Pressure; eGFR: estimated Glomerular Filtration Rate; FPG: Fasting Plasma Glucose; HbA1c: Haemoglobin A1c; HDL-c: HDL cholesterol; SBP: Systolic Blood Pressure; T-c: total cholesterol, Group A: patients with normal glucose levels; TG: triglycerides. Group B: patients with altered glucose levels. Data are expressed as mean ±SD.

Baseline clinical and metabolic characteristics of patients according to glucose levels at follow-up

The median time of observation was 6.0±1.6 years (5-15 years). In group A, 169 patients (AA: 146 on therapy, 23 controls) maintained normal glucose levels, 42 (AB: 38 on therapy, 4 controls) developed altered glucose levels and 5 (AC: 1 on therapy, 4 controls) had NOD. Conversely, in group B, 18 patients (BA: 13 on therapy, 5 controls) returned to normal glucose levels, 37 (BB: 30 on therapy, 7 controls) maintained altered glucose levels and 37 (BC: 36 on therapy, 1 control) developed NOD. The cumulative incidence of NOD during follow-up was 13.6% (11.6% in controls and 13.9% in treated patients). NOD was diagnosed after a median time of 3.4±1.8 years after the first visit.

All the AA subjects presented lower BMI (24.1±3.3 vs 26.1±3.4 Kg/m^2; p<0.001), waist circumference (81.2±10.7 vs 97.8±9.0 cm; p<0.001), SBP (126.5±16.8 vs 134.4±13.3 mmHg; p<0.001), DBP (78.0±8.1 vs 80.9±8.6 mmHg; p<0.020), glucose (86.1±7.9 vs 91.1±6.8 mg/dl; p<0.001), while they had higher HDL-c levels (61.1±17.5 vs 55.9±15.7 mg/dl p<0.038) than in group AB. AA subjects also had lower BMI (24.1±3.3 vs 28.4±3.5 Kg/m^2; p<0.004) and higher T-c (274.8±54.7 vs 242.2±102.1 mg/dl; p<0.041) and HDL-c levels (61.1±17.5 vs 47.0±12.3 mg/dl, p<0.038) than AC subjects at baseline. Furthermore, the AA group as a whole and as a treatment group presented a lower prevalence of obesity (p<0.003), thiazide diuretics intake (p<0.033) and family history of type 2 diabetes (p<0.021) and a higher prevalence of family history of dyslipidemia (p<0.014) than AB plus AC subjects.

The BB group had lower BMI (26.3±4.8 vs 28.5±4.0 Kg/m^2; p<0.004), waist circumference (91.3±11.7 vs 103.8±5.4 cm; p<0.006), glucose levels (109.7±6.7 vs 115.5±9.1 mg/dl; p<0.001), HbA_{1c} (5.8±0.3 vs 6.2±0.4%; p<0.001), LDL-c to HDL-c ratio (3.0±1.0 vs 3.7±1.8; p<0.020), T-c to HDL-c ratio (4.6±1.2 vs 5.6±2.6; p<0.009), and higher HDL-c (57.1±19.5 vs 48.2±12.5 mg/dl; p<0.004) than BC. The BB group had higher SBP (135.3±18.0 vs 125.5±12.4 mmHg; p<0.029), glucose (109.7±6.7 vs 106.1±4.5 mg/dl; p<0.014), TG (177.6±98.6 vs 144.4±109.5 mg/dl; p<0.040), AST (24.9±6.6 vs 20.4±5.7 IU/L; p<0.010) and ALT levels (28.8±9.2 vs 20.9±9.1 IU/L; p<0.001) than BA at baseline.

In addition, the AB group had lower weight (p<0.020), BMI (p<0.001), SBP (p<0.002), glucose (p<0.0001), HbA_{1c} (p<0.018), ALT (25.8±11.8 vs 32.7±10.9 UI/L p<0.001), and higher T-c (274.5±53.5 vs 258.5±50.9 mg/dl p<0.041) and HDL-c levels (p<0.011) than BC.

Risk factors associated with the development of altered glucose levels

Risk factors associated with altered glucose levels and NOD

By evaluating the whole population, family history of type 2 diabetes (OR: 3.1, 95% CI 1.3-7.2, p<0.006),

BMI >30 kg/m^2 (OR: 5.2, 95% CI 1.5-17.8, p<0.006), glucose levels >100 mg/dl (OR: 22.6, 95% CI 8.5-59.6, p<0.006), treatment with statins (OR: 4.0, 95% CI 1.1-14.1, p<0.029) increased, whereas no alcohol consumption (OR: 0.4, 95% CI 0.2-0.9, p<0.025) decreased, the risk of maintaining/developing altered glucose levels or developing NOD. Thiazide intake was marginally significant (OR: 14.1, 95% CI 0.9-210.4, p=0.052). The intake of statins (OR: 4.0, 95% CI 1.1-14.1, p<0.029), and in particular simvastatin and atorvastatin, was associated with increased risk in the full custom models (Table 3). By using the number of TNT factors the presence of increasing TNT risk factors increased the risk (p<0.0001).

In group A, a family history of type 2 diabetes (OR: 3.4, 95% CI 1.3-8.9, p<0.010), BMI >30 kg/m^2 (OR: 8.5, 95% CI 2.0-35.8, p<0.003), thiazide therapy (OR: 21.9, 95% CI 1.2-384.2, p<0.034) increased, whereas no alcohol consumption (OR: 0.3, 95% CI 0.1-0.8, p<0.010) reduced, the risk of developing altered glucose levels or NOD. No effects of statins (OR: 1.1, 95% CI 0.4-3.4, p=0.753) were seen in any of the models. In group B, hypertension (OR: 5.0, 95% CI 1.0-25.3, p<0.049) and hypertriglyceridemia (OR: 3.5, 95% CI 1.0-11.8, p<0.040) increased the risk of maintaining altered glucose levels or developing NOD. Treatment with statins (OR: 7.5, 95% CI 1.5-37.4, p<0.012), and in particular atorvastatin, was associated with the increased risk in the full custom models (Table 3).

Risk factors associated with NOD

The analysis was performed in group B due to the relatively few cases of NOD in group A by the end of follow-up (4 controls and 1 treated patient).

In group B, having a BMI >30 Kg/m^2 (HR: 2.7, 95% CI 1.2-6.0, p<0.009) and hypertension (HR: 2.5, 95% CI 1.1-5.3, p<0.021) increased the risk of developing NOD. Statin therapy did not reach significance with respect to diet, possibly due to the sample size (37 events censored on statin and 1 event censored on diet therapy).

DISCUSSION

In our retrospective study on a cohort of dyslipidemic patients treated with statins or diet and with a follow-up longer than 5 years, those who developed or maintained altered glucose levels or NOD had a poor metabolic phenotype at baseline. Statin therapy, in particular atorvastatin, was associated with altered glucose levels or NOD in those who had altered fasting glucose at baseline, suggesting that this population should be carefully monitored.

The cumulative incidence of NOD was 13.6%, 11.6% in controls and 13.9% in those treated with statins.

A meta-analysis of 13 randomized controlled trials with >90,000 participants found a 9% risk of incident NOD after 4 years of statin treatment, particularly in

Table 3. Risk to maintain altered glucose levels or to have new onset diabetes in logistic regression.

	All subjects			Group A			Group B		
	OR	**CI95%**	**p**	**OR**	**CI95%**	**p**	**OR**	**CI95%**	**p**
Diet	1.000			1.000			1.000		
Prava+Fluva	2.045	0.417-10.023	0.378	0.566	0.205-2.383	0.698	1.750	0.385-7.951	0.469
Simvastatin	5.797	1.434-23.438	0.010	0.898	0.318-2.534	0.898	5.625	0.894-35.389	0.067
Atorvastatin	4.343	1.024-18.422	0.042	0.975	0.314-3.027	0.966	7.812	1.262-48.356	0.023
Rosuvastatin	3.851	0.807-18.365	0.911	0.886	0.300-2.616	0.827	1.875	0.396-10.463	0.474

CI: confidence interval; OR: odds ratio; Prava+Fluva: pravastatin+ fluvastatin; Group A: subjects (216) with normal glucose levels at baseline; Group B: subjects (92) with altered glucose levels at baseline. The models also included the following fixed factors and covariates: age, sex, BMI >30 Kg/m^2, hypertension, hypertriglyceridemia, thiazide, PUFA-n3 or ezetimibe assumption, alcohol consumption, smoking, years of follow-up for Group A and B, and also for glucose >100 mg/dl for all subjects.
Table 3 represents OR only for statin assumption (independent variable).

those who were older[4] and on intensive-dose statin therapy.[5] The higher incidence in our cohort could be explained by the longer follow-up (median: 6 years, range 5-15 years). Furthermore, because our patients were referred to a tertiary referral center for dyslipidemia, a selection bias due to a higher risk metabolic phenotype cannot be excluded. In addition, our patients were subjected to a careful staging of CV risk factors and an OGTT was also performed for any increase in fasting plasma glucose. Moreover, the incidence of NOD in patients treated with diet was higher than that reported in other studies in European countries.[17,18] This difference could be due to difficulties in the ascertainment of the incidence of NOD in the general population and dyslipidemic patients could be a population at higher risk when diet adherence is not good, as shown in the "Primary prevention of cardiovascular disease with pravastatin in Japan" (MEGA) study.[19]

Cederberg et al[18] published similar data based on the follow-up of the METabolic Syndrome In Men (METSIM) study conducted in men randomly selected from the Kuopio, Finland, population register. They observed an increased risk of NOD in those treated with statins, but also a worsening of hyperglycemia at oral glucose tolerance test (OGTT) that represented a progressive prediabetic state. We confirm this observation in a selected population of dyslipidemic patients of both sexes on primary prevention and with a longer follow-up. The most important finding of our study is that the glucose phenotype at baseline was crucial in predicting the risk of developing altered glucose levels with statins, whereas the risk was not present when fasting glucose levels were <100 mg/dl (5.6 mmol/l). A *post hoc* analysis of the patients without diabetes at baseline from both the TNT and IDEAL trials reported similar findings.[20]

Our analysis of anthropometric and metabolic parameters showed that patients who developed altered glucose levels or NOD had an insulin resistance phenotype at baseline. In particular, they had more central obesity and hypertension as well as lower HDL-c levels and maintained these characteristics over time. These findings suggest that statins could more rapidly worsen a metabolic phenotype already known to predict the risk of NOD.[19,21,22] Because we also observed that a higher number of TNT risk factors increased the risk, clinicians should carefully monitor patients with this phenotype since a synergistic action of statins with other diabetes risk factors is likely.

A family history of type 2 diabetes is recognized as a risk factor for NOD development. It has been demonstrated that the risk is three times higher in people who have a relative affected by type 2 diabetes and 6 times higher in people who have both parents affected.[23,24] In our series, a family history of type 2 diabetes should be another factor to consider in the global management of patients treated with statins.

Alcohol abuse has been identified as a possible risk factor for NOD since it results in an extreme intake of carbohydrates, obesity, increasing pancreatitis incidence and worsening of hepatic function together with an alteration of glucose metabolism.[25] Interestingly, although we were unable to stratify daily alcohol intake due to the retrospective nature of the study, no alcohol consumption was protective. This factor should be considered in the follow-up of patients treated with statins. Studies with dose-response analyses are needed, especially since it was recently observed that a moderate reduction risk of NOD is present but confined to women with moderate levels of alcohol consumption.[26]

Moreover, we showed that statins, mainly atorvastatin, are linked to a higher risk of NOD or altered glucose levels in agreement with the majority of the literature. Pravastatin and pitavastatin even showed a protective effect on glucose alteration development.[27-29] The reason why some, but not all, statins have detrimental effects on glucose metabolism remains unresolved. Beyond molecule-dependent mechanisms, other possible explanations include residual confounding factors, including a different lifestyle and an aggressive statin treatment.

We also reported a lower risk of having altered glucose levels at baseline, before any treatment with statins, in those with a family history of dyslipidemia. These results are in line with several observational studies that reported an inverse association between familial hypercholesterolemia and the risk of type 2 diabetes with a certain genotype-phenotype correlation.[30] Moreover, the SAFEHEART cohort study showed that statins do not increase the risk of type 2 diabetes mellitus in patients with familial hyper-

cholesterolemia.[31] These data point to the intriguing hypothesis that LDL receptor (LDLR) mutations can improve the function of pancreatic β cells.[30]

The current study has some limitations. First, due to its retrospective nature we could not control for adherence to treatment. Secondly, because the cases of altered glucose levels could not be ascertained in registries or with medical prescriptions, Cox regression models for this condition were not performed. Additionally, we studied only a few controls, but this is linked to the nature of a population followed in a tertiary referral center. Conversely, the study included a number of patients with a follow-up >5 years. Finally, many risk factors including family history of type 2 diabetes were considered.

In conclusion, this study confirmed that statins are associated with an increased risk of NOD or altered glucose levels, mainly in subjects with fasting glucose >100 mg/dl before initiating treatment. A worse metabolic phenotype, lifestyle risk factors and family history of type 2 diabetes contributed to the risk.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Research paper

Hormonal responses following eccentric exercise in humans*

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ABSTRACT

OBJECTIVE: Mechanically overloaded muscle and its subsequent damage are strong stimuli for eliciting acute hormonal changes, while the muscle adaptation which occurs following exercise-induced muscle damage may involve complex hormonal responses before the completion of muscle regeneration. The purpose of this study was to investigate systemic responses of various hormones, as well as secreted proteins that are exercise-regulated and associated with muscle adaptation, for several days after eccentric exercise-induced muscle damage in humans. DESIGN: Nine young male volunteers performed 50 maximal eccentric muscle actions using the knee extensor muscles of both legs. Blood samples were drawn before and at 6, 48 and 120 hours post exercise and serum levels of growth hormone (GH), insulin-like growth factor binding protein-3 (IGFBP-3), cortisol, prolactin, thyroid-stimulating hormone (TSH), free thyroxine (fT4), irisin, follistatin and sclerostin were measured. Myoglobin (Mb) concentration and lactate dehydrogenase (LDH) activity were also evaluated as indirect markers of muscle damage. RESULTS: Significant alterations in Mb and LDH were observed over time after eccentric exercise (p=0.039-0.001). A late serum increase in fT4 and decrease in irisin levels, along with an early and persistent decrease in IGFBP-3 levels, were observed following the muscle-damaging exercise (p=0.049-0.016). GH, cortisol, prolactin, TSH, follistatin and sclerostin exhibited moderate changes during the recovery period after exercise, though without reaching statistical significance (p>0.05), while correlational analyses revealed significant associations between GH and IGFBP-3, prolactin and sclerostin over time (p=0.049-0.001). CONCLUSIONS: The significant hormonal responses observed in this study may indicate their involvement in the regenerative mechanisms following muscle damage, potentially as part of a regulatory network to support a normal adaptation process after muscle-damaging exercise.

Key words: Follistatin, IGFBP-3, Irisin, Muscle damage, Prolactin, Sclerostin, TSH, T4

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INTRODUCTION

Skeletal muscle overloading and overstretch, or their combination, that occur in eccentric muscle actions have been extensively shown to result in muscle damage, thus eccentric exercise has been utilized as a well characterized model to study contraction-induced muscle damage and its consequent responses.[1-3] Eccentric exercise-induced muscle damage has been associated with structural and functional disturbances in the exercised muscle, with changes in its mechanical properties, loss of muscle fiber integrity and leakage of muscle proteins into the blood being some well characterized responses to muscle damage.[4-7]

Mechanical overloading of muscle and its subsequent damage appear to be strong stimuli for eliciting significant acute hormonal responses, which may be more critical to muscle tissue repair, remodelling and growth than chronic exercise-induced changes in hormonal levels at rest.[8,9] Moreover, hormones are sensitive to exercise-induced stress and play various roles in the anabolic/catabolic and metabolic drive in muscle. Thus, muscle regeneration and adaptation which occur following exercise-induced muscle damage are likely to involve complex systemic responses of hormones and muscle-associated secreted proteins before the completion of muscle regeneration. Such potential interactions between hormones and other secreted proteins could therefore be part of an integrated system of signalling to mediate and support physiological adaptations to muscle mechanical overloading and damage,[9] hence, the net result of eccentric exercise-induced hormonal, metabolic and anabolic responses appears to be a novel adaptive signal within the muscle.[10]

However, although much has been learned about the local mechanisms and the cellular and molecular interactions that mediate muscle regeneration and adaptation following exercise-induced muscle damage,[11-14] the role of systemic, and particularly hormonal, responses to muscle damage and its subsequent adaptations is still not fully defined. Only a limited number of studies have examined the acute exercise-induced hormonal responses following mechanical overloading (resistance exercise) or muscle-damaging eccentric exercise in humans,[9,15-17] while in those studies only few hormones, such as cortisol, testosterone or growth hormone (GH), have been investigated and this only for a short period of time, i.e. less than one hour, after exercise.

Thus, the challenge remains to further characterize the simultaneous systemic responses of various hormones and muscle-associated proteins during the regeneration process after muscle-damaging exercise in the context of their potential interactions in the regulation of muscle damage-induced adaptation. The purpose of this study was therefore to examine the eccentric exercise-induced responses of hormones[8] as well as secreted proteins that are exercise-regulated and have been associated with skeletal muscle regeneration and adaptation,[18-20] i.e. GH, insulin-like growth factor binding protein-3 (IGFBP-3), cortisol, prolactin, thyroid-stimulating hormone (TSH), free thyroxine (fT4), irisin, follistatin and sclerostin. Indeed, some of these hormones have aroused particular interest because of their potential role in exercise-induced muscle adaptations.[19-24] In particular, irisin is a hormone predominantly expressed in human muscle[25,26] and is required for the exercise-induced conversion of white adipose tissue to brown.[18,25] In addition, follistatin is an antagonist of myostatin, the negative regulator of muscle regeneration,[27] while sclerostin is a mechanical loading-regulated protein whose serum levels have been shown to be affected by physical exercise.[20,28]

We monitored the serum changes of the aforementioned hormones and muscle-associated secreted proteins for several days after the completion of an eccentric exercise protocol in humans, posing the hypothesis that their circulating levels would be changed during the recovery period following exercise-induced muscle damage, thus suggesting their potential role as mediators of muscle regeneration and adaptation after the damaging exercise.

MATERIALS AND METHODS

Ethical approval

All volunteers provided written informed consent to participate in this study, which was approved by the Ethics Committee of the National and Kapodistrian University of Athens, while all experimental procedures conformed to the Declaration of Helsinki.

Subjects

Nine healthy men (age 25.7 ± 1.7 years, height 180.4 ± 1.7 cm, body mass 77.2 ± 2.7 kg, body mass index 23.7 ± 0.6) participated in the study. The participants were physically active but had not participated in any type of resistance training or regular exercise regime for at least 6 months before the study and also were unaccustomed to high-intensity eccentric exercise. These volunteers were free of any lower extremity musculoskeletal disorders; they also refrained from taking any nutritional supplementations or medications throughout the experimental period. In addition, the subjects were not allowed to perform any vigorous physical activities during the entire experimental period. They were also instructed to maintain their habitual diet and on the day prior to and the day of each blood draw to have equivalent meals.

Experimental design

The participants performed a maximal eccentric exercise protocol of the knee extensors with each leg. Before and at 6, 48 and 120 hrs after the eccentric exercise, blood samples were collected from each individual volunteer. The blood sampling time-points were chosen to cover an adequate period within the regenerative phase following exercise-induced damage.[29]

Eccentric exercise protocol

The subjects performed an eccentric exercise bout with the knee extensors of each leg on an isokinetic dynamometer (Cybex Norm Lumex, Inc., Ronkonkoma, NY, USA), which has been shown to result in muscle damage.[3] Briefly, before the exercise protocol the subjects completed a familiarization session in which they were acquainted with the procedure of the eccentric exercise with each leg. The exercise protocol consisted of 2 sets of 25 maximal voluntary eccentric (lengthening) muscle actions in isokinetic mode, while a 5-min break was allowed between the sets.

Blood sampling and serum analyses

Blood samples were withdrawn prior to and after the exercise bout (at 6, 48 and 120 hrs post exercise) and at the same time of the day for all subjects. The participants were seated quietly for 30 min and 10 ml of blood were drawn. Blood samples allowed to clot at room temperature for 30 min and serum was collected after centrifugation at 4,000 RPM for 10 min at 4°C, stored frozen in 0.5 ml aliquots at -80° C and only thawed once for analysis. Serum myoglobin (Mb) concentration and lactate dehydrogenase (LDH) activity were assessed as indirect markers of muscle damage. In particular, measurement of LDH activity was performed using an automated commercially available kit (Roche Diagnostics, Mannheim, Germany) in a Roche/Hitachi ACN 057 (Roche, Mannheim, Germany) at 37°C, while myoglobin concentrations were determined using an immunoturbidimetric assay (Turbiquant, Dade Behring, Marburg, Germany).

Serum GH, insulin-like growth factor binding protein-3 (IGFBP-3), cortisol, prolactin, thyroid-stimulating hormone (TSH), free thyroxine (fT4), irisin, follistatin and sclerostin were determined by standard sandwich enzyme-linked immunosorbent assay (ELISA) protocols using commercially available kits (GH and cortisol: Enzo Life Sciences, NY, USA; IGFBP-3, follistatin and sclerostin: Quantikine HS, R&D Systems inc., MN, USA; prolactin: Cayman Chemical, Michigan, USA; TSH and fT4: MP Biomedicals, NY, USA; irisin: Adipogen, Switzerland) according to the manufacturer's instructions. The colour formation was measured by a microplate reader (Versamax, Molecular Devices, CA, USA) at 450 nm and calculations were carried out using SoftMax Pro software (Molecular Devices, CA, USA). All samples were run simultaneously, analyzed in duplicate and the results were averaged. According to the manufacturers, the minimal detection limits of the assays used were 0.93 pg ml^{-1}, 56.72 pg ml^{-1}, 0.05 ng ml^{-1}, 0.12 ng/ml, 0.05 μIU ml^{-1}, 0.054 ng dl^{-1}, 1 ng ml^{-1}, 83 pg ml^{-1}, 3.8 pg ml^{-1} for GH, cortisol, IGFBP-3, prolactin, TSH, fT4, irisin, follistatin and sclerostin, respectively, while the intra- and inter-assay coefficient of variation (CV) were as follows: 4.0% to 4.2% and 1.9% to 6.4% for GH, 7.3% to 10.5% and 7.8% to 13.4% for cortisol, 2.3 % to 5.0% and 5.4% to 8.0% for IGFBP-3, 2.8% to 4.1% and 4.6% to 5.5% for prolactin, 6.4% to 9.2% and 7.6% to 12.9% for TSH, 3.3% to 10.9% and 6.0% to 10.8% for fT4, 4.9% to 8.2% and 8.1% to 9.7% for irisin, 2.0% to 2.7% and 7.1% to 9.2% for follistatin and 1.8% to 2.1% and 8.2% to 10.8% for sclerostin.

Statistical analysis

One-way analysis of variance (ANOVA) with repeated measures over time was used to evaluate changes in all serum measurements (SPSS v. 22 statistical package). A non-parametric (Friedman) test was conducted where the data had violated the assumptions necessary to run the repeated measures one-way ANOVA (e.g., data not normally distributed). Where a significant F ratio was found for main effect ($p<0.05$), the means were compared using Dunnett's post-hoc test or Wilcoxon signed-rank test with Bonferroni correction for non-parametric tests. Pearson's correlation coefficient (r) was used to determine correlations between variables. All data are presented as mean ± standard error of the mean (S.E.M). The level of significance was set at $p<0.05$.

RESULTS

Significant alterations in markers of muscle damage were observed after eccentric exercise. Specifically, LDH activity levels were increased significantly ($F=3.99$, $df=3$) at 6 ($p=0.003$) and 48 hrs ($p=0.019$) following muscle-damaging exercise and remained elevated up to 120 hrs post exercise. Myoglobin concentrations were significantly increased ($F=12.94$, $df=3$) at 6 ($p<0.001$) and 120 hrs ($p=0.039$) post exercise, while both LDH and Mb showed their peak response at 6 hrs post exercise (Figure 1).

Standard curves of the ELISA analyses (absorbance vs concentration) of all the factors examined had an R^2 coefficient ranging between 0.971 and 1. Systemic responses of the various hormones and muscle-associated secreted proteins after the muscle-damaging exercise are shown in Table 1. GH, cortisol and prolactin showed moderate increases following eccentric exercise, which peaked at 6 hrs for GH and cortisol and at 48 hrs for prolactin, however without reaching statistical significance due to a large variability shown between the subjects' responses ($p>0.05$; Table 1). TSH increased gradually but not significantly up to 120 hrs post-exercise ($p>0.05$), while fT4 also exhibited gradual increases post exercise, which became significant ($F=2.77$, $df=3$) at 48 ($p=0.016$) and 120 hrs ($p=0.049$) following eccentric exercise (Table 1).

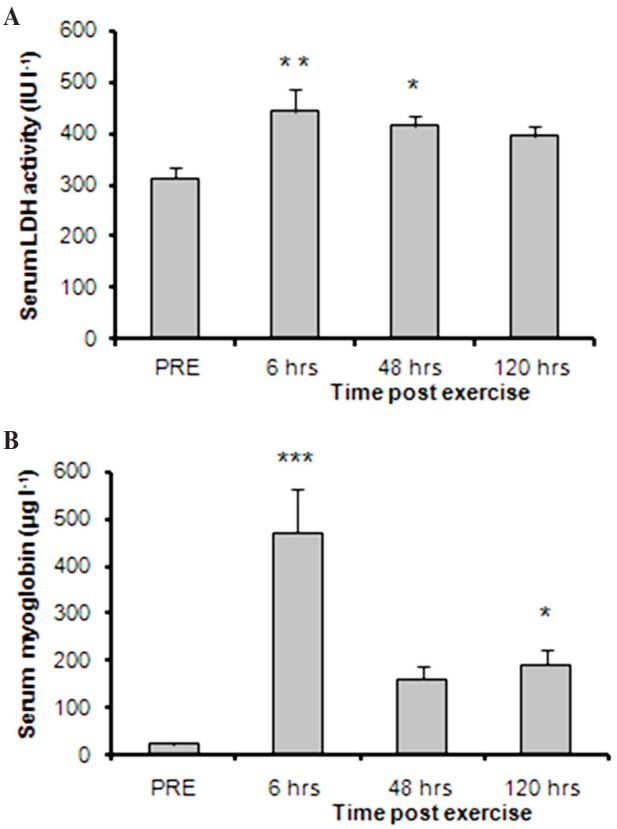

Figure 1. Changes in serum (A) lactate dehydrogenase (LDH) activity and (B) myoglobin (Mb) concentration over time compared with the pre-exercise (PRE) levels (mean ± S.E.M.; n=9). Significantly different from pre-exercise; *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

Interestingly, serum levels of both IGFBP-3 and irisin decreased post exercise and they were significantly lower ($F=2.14$, $df=3$) for irisin at 120 hrs post exercise compared with the baseline (PRE) levels ($p=0.032$; Table 1). Furthermore, when the post-exercise responses were analyzed as percent changes in relation to baseline levels significant changes were revealed over time for IGFBP-3, irisin and fT4 (Figure 2). In particular, IGFBP-3 decreased significantly throughout the post-exercise period ($p<0.011 – 0.001$), irisin showed a significant decrease 120 hrs ($p=0.003$) after exercise, while fT4 exhibited a gradual increase following eccentric exercise, reaching statistical significance at 48 ($p=0.004$) and 120 hrs ($p=0.009$) post exercise (Figure 2).

Serum levels of follistatin peaked at 48 hrs, showing a more than 60% increase before returning to baseline levels at 120 hrs post exercise, while sclerostin

Table 1. Serum concentrations of various hormones and muscle-associated secreted proteins examined at baseline and 6, 48 and 120 hrs after muscle damaging eccentric exercise. Values represent the means (± S.E.M), (n=9)

	PRE	6 hrs	48 hrs	120 hrs
GH (pg/ml)	495.6 (± 152.8)	726.1 (± 169.1)	665.1 (± 217.1)	510.4 (± 152.8)
IGFBP-3 (ng/ml)	1391.9 (± 8.0)	1216.2 (± 7.0)	1236.2 (± 7.1)	1247.7 (± 8.0)
Cortisol (ng/ml)	267.0 (± 40.8)	337.4 (± 77.3)	321.4 (± 60.0)	312.1 (± 43.0)
Prolactin (ng/ml)	23.4 (± 3.1)	28.1 (± 4.7)	30.2 (± 4.1)	25.7 (± 4.6)
TSH (μIU/ml)	1.09 (± 0.14)	1.27 (± 0.15)	1.17 (± 0.20)	1.33 (± 0.17)
fT4 (ng/dl)	1.13 (± 0.02)	1.20 (± 0.05)	1.29 (± 0.04)* (p=0.016)	1.26 (± 0.05)* (p=0.049)
Irisin (μg/ml)	0.22 (± 0.03)	0.20 (± 0.02)	0.17 (± 0.01)	0.14 (± 0.02)* (p=0.032)
Follistatin (pg/ml)	2080.2 (± 200.3)	2827.2 (± 472.6)	2924.4 (± 330.2)	2144.4 (± 177.9)
Sclerostin (pg/ml)	1734.0 (± 639.1)	1432.8 (± 491.5)	1280.5 (± 533.4)	2239.1 (± 1066.1)

GH: Growth hormone; IGFBP-3: Insulin-like growth factor binding protein-3; TSH: Thyroid-stimulating hormone; fT4: Free thyroxine.
*: Significantly different compared with the pre-exercise (PRE) values.

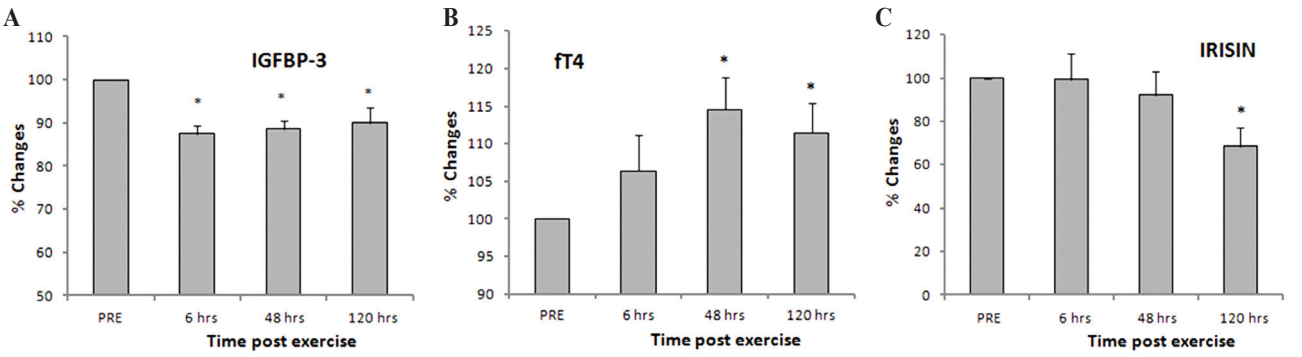

Figure 2. Percent changes of serum (A) insulin-like growth factor binding protein-3 (IGFBP-3), (B) free thyroxine (fT4), and (C) irisin over time in relation to their pre-exercise (PRE) levels (mean ± S.E.M.; n=9). Significantly different from pre-exercise; *: $p < 0.05$.

exhibited a reverse pattern of response over time, decreasing gradually before exhibiting a late mild increase at 120 hrs post eccentric exercise, though those changes failed to reach significance due to a large individual variation in the circulating levels of these secreted factors ($p>0.05$; Table 1).

Correlation analyses revealed significant positive associations over time between GH and prolactin ($r=0.585 – 0.671$; $p=0.037 – 0.010$), as well as between GH and sclerostin ($r=0.560 – 0.852$; $p=0.046 – 0.001$). Moreover, a significant negative correlation was found between GH and IGFBP-3 serum levels over time [$r= -0.420 – (-0.537)$; $p=0.049$].

DISCUSSION

Our study examined the changes in the circulating levels of various hormones and secreted proteins associated with skeletal muscle regeneration and adaptation up to 5 days after an eccentric exercise protocol in order to reveal their potential involvement and interactions in the regeneration process following exercise-induced muscle damage. The remarkable and sustained changes in the indirect markers of damage, i.e. the leakage of muscle proteins Mb and LDH into circulation post exercise, indicated that the eccentric exercise used in this study did result in muscle damage. Our main findings demonstrated

a late serum increase in fT4 and decrease in irisin levels, along with an early and persistent decrease in IGFBP-3 levels after exercise. To the best of the authors' knowledge, this is the first study to show significant systemic responses of T4 and irisin during the regeneration period following muscle-damaging exercise, indicating the involvement of those hormones in the regenerative and adaptive mechanisms following muscle damage. Furthermore, the present study revealed moderate systemic increases in TSH, GH, cortisol and prolactin over time, while circulating levels of secreted proteins follistatin and sclerostin also exhibited modest alterations post exercise.

An exercise bout represents physical stress for the body and induces an acute disturbance of its homeostasis, particularly in the exercised muscles but also in other cells and organs, while in the recovery phase homeostasis is re-established.[30,31] Specifically, regulatory mechanisms appear to be quickly reengaged after a mechanical overloading (resistance) exercise workout so that homeostasis is restored within 1 hour post exercise.[8] However, following muscle-damaging exercise, a longer, highly orchestrated sequence of responses is activated, initially associated with an inflammatory and degenerative phase at the site of damage and followed by the myogenic differentiation and fusion of myoblasts to complete muscle regeneration.[29]

Although the role of thyroid hormones during muscle regeneration is not as yet fully defined, it is well known that skeletal muscle is a major target of thyroid hormone signalling while, further, this signalling regulates crucial biological functions, including energy expenditure, development and growth. In particular, expression of the thyroid hormone converting enzyme type 2 iodothyronine deiodinase (DIO2), which converts the prohormone T4 to the active hormone T3, is increased in developing or injured muscle and provides the potential for local control of the uptake of T4 as well as of its activation and inactivation within skeletal muscle.[24] Moreover, there is compelling evidence of a functional link between DIO2 induction and myogenic differentiation[24] and also of an essential role of thyroid hormone signalling in muscle regeneration after injury.[23,32] Hence, the late elevated circulating levels of fT4, and to a lesser extent of TSH, found in the present study during the recovery period after muscle damage appear to corroborate the aforementioned functional interactions between thyroid hormones and muscle regeneration and further suggest the potential contribution of the circulating thyroid hormones, especially of thyroxine, to muscle regeneration following damaging exercise. Nevertheless, the importance of fT4 increase post exercise needs further investigation, since the main effect of thyroid hormones on myocyte growth and metabolism is exerted by the active hormone T3. Moreover, the increased circulating fT4 levels could be a result of a decreased systemic (from the liver and/or kidney) activity of DIO2, which might result in normal serum levels of T3. In addition, the increased, though not significantly, cortisol levels found in this study could also lead to the inhibition of peripheral conversion of T4 to T3.

On the other hand, thyroid hormones are influencing factors in controlling energy balance and their signalling can modulate metabolic rate by either decreasing metabolic efficiency or by uncoupling ATP synthesis in the mitochondria of skeletal muscle. Indeed, increased mitochondrial uncoupling in muscle and raised energy expenditure have been exhibited by individuals who had been treated with a T4 synthetic analogue and also by patients who had elevated serum thyroid hormone levels.[33,34] Moreover, recent studies may have,[29] or may have not revealed[30] an association between thyroid hormones and the muscle-derived circulating protein, irisin, in thyrometabolic abnormalities. Irisin, apart from being required for the exercise-induced conversion of white adipose tissue to brown,[18,25] promotes mitochondrial uncoupling in brown adipose tissue, thereby pointing to an important indirect role of muscle in the regulation of energy expenditure.[24] Thus, it could be postulated that the gradually decreased circulating levels of irisin observed in the present study could influence adipose tissue metabolism and insulin resistance[35] during the recovery period following muscle-damaging exercise. At this point it should be mentioned that there are claims that many human irisin antibodies used in commercial ELISA kits lack required specificity and that when this hormone was measured by the precise mass spectrometry method[36] considerably lower concentrations of circulating irisin were detected (i.e.,

~3.6-4.3 ng/ml) compared to the results of our study and others. It is thus worth noting this limitation of our study, although it was clearly stated that irisin unequivocally circulates and is regulated by exercise in humans.[36] Taking into account all the above, we might speculate that the significantly decreased levels of irisin concurrently with the increased circulating fT4 levels may reflect a compensatory mechanism to counterbalance the raised energy expenditure expected to be induced by the elevated levels of T4 during regeneration of the damaged muscles.[33,34]

The final outcome of the multiple events that take place following muscle damage is also influenced by a crucial balance between, among others, anabolic and catabolic factors. Nevertheless, less is known about the systemic responses of competitive (anabolic vs catabolic) hormones, such as GH and cortisol, during regeneration after muscle damage. Previous studies examining the more acute hormonal responses to mechanical overloading or muscle-damaging exercise have shown significant increases of those hormones during the next hour post exercise.[15-17] Such early responses may reflect the metabolic and/or physiological stress of exercise, whereas the late hormonal changes in the circulation are reflective of tissue homeostasis mechanisms involving protein metabolism and tissue repair during the recovery process.[8,37]

The findings of our study suggest that eccentric exercise triggers only moderate adaptive changes of the anabolic/catabolic and physiological stress-regulated hormones,[30] while the positive relationship found between GH and prolactin over time imply a systemic counterregulation of the anabolic and catabolic processes during the adaptation following muscle-damaging exercise.

Further, in this context, this study was the first focusing on the systemic responses of follistatin after exercise-induced muscle damage. Follistatin has been shown to promote myogenic differentiation *in vitro*, as well as hypertrophy and muscle regeneration after injury *in vivo*, through myostatin blockage but also via myostatin-driven independent mechanisms.[19,21,38] We revealed a pattern of response of follistatin similar to that of GH. Although these increases in the levels of follistatin were not significantly high, they might imply the potential involvement of circulating follistatin as well in the regenerative and or anabolic adaptations following exercise-induced muscle damage, which is in accordance with recent findings regarding the therapeutic effects of systemic follistatin administration on muscle regeneration.[22]

Another interesting finding of our study was the decreased serum IGFBP-3 levels throughout the 5-day recovery period after exercise, which were negatively correlated with the corresponding GH levels. In general, IGFBPs and particularly IGFBP-3 can modulate the IGF-dependent effects, both in the circulation and in the extracellular environment, via regulation of IGF-1 concentration and its local bioavailability in the tissue.[39] We previously did not find any significant changes in serum IGF-1 levels throughout a period of 16 days following muscle-damaging exercise.[12] However, less is known concerning late IGFBP-3 systemic responses after exercise-induced muscle damage. Another study showed that circulating levels of IGFBP-3 were only increased for the first hour after a mechanical overloading exercise and further suggested that the acute effect of a resistance exercise bout is not the changes in circulating IGF-1 levels but rather the manner in which this anabolic factor is partitioned among its binding proteins.[40] Moreover, a potentiation of IGF activity by IGFBP-3 has been demonstrated in many in vitro systems, leading to the concept that cell association and processing of IGFBP-3 to a form of reduced affinity to IGF-1 is involved in the enhancement of the availability of IGF-1 to its receptor(s),[41,42] while, further, GH induces IGFBP-3 local accumulation in cells.[43] Hence, we could speculate that GH and IGFBP-3 responses and their negative correlations found in this study may indicate a GH-induced tissue accumulation of circulating IGFBP-3 as a mechanism of potentiating the regenerative and anabolic effects of IGF-1 following muscle damage.[12,44,45]

Lastly, we explored the potential responses of sclerostin, which is secreted by osteocytes and acts as an inhibitor of bone formation and whose serum levels appear to be affected by physical exercise.[20,28] In particular, 8 wk of exercise training resulted in significantly decreased serum levels of sclerostin; however, there are no reports in the literature regarding the acute effects of muscle mechanical overloading and damage

on circulating levels of this secreted factor. Interestingly, in this study we observed a gradual decrease of circulating sclerostin up to 48 hrs after the single bout of eccentric exercise, before increasing (though without reaching significance) at 120 hrs post exercise, accompanied by its strong positive correlations with GH responses. More studies are needed to identify the potential interactions between muscle-damaging exercise and acute systemic responses of sclerostin.

In conclusion, the significant systemic hormonal responses observed in this study may point to their involvement in the regenerative mechanisms following muscle damage. It can be assumed that hormones and secreted factors released into the circulation act as part of a regulatory network to support a normal adaptation process following muscle-damaging exercise. Further studies are required to characterize the mechanisms by which hormonal responses are triggered and regulated at the systemic level during recovery after exercise-induced muscle damage. Moreover, it would be of particular interest to investigate the end organ effects of the above hormonal alterations on target organs other than the damaged skeletal muscle, thus revealing potential clinical implications of this particular type of exercise.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Research paper

Diagnostic value of the water deprivation test in the polyuria-polydipsia syndrome

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ABSTRACT

OBJECTIVE: Diabetes insipidus (DI) and primary polydipsia (PP) are characterised by polyuria and polydipsia. It is crucial to differentiate between these two disorders since the treatment is different. The aim of this study was to evaluate the diagnostic value of the short and an extended variant of the water deprivation test (WDT) and of measuring urinary vasopressin (AVP) in patients with polyuria and polydipsia. DESIGN: A retrospective, single-centre study based on WDTs performed between 2004 and 2014 including 104 consecutive patients with the polyuria-polydipsia syndrome. During a strict water deprivation, weight, urinary osmolality, urinary vasopressin and specific gravity were collected until one of the following was reached: i) >3% weight reduction, ii) Urinary specific gravity >1.020 or, urinary osmolality >800 mOsm/L, iii) Intolerable adverse symptoms such as excessive thirst. RESULTS: Out of 104 patients (67 women, 37 men), 21 (20%) were diagnosed with DI and 83 (80%) with PP. The median (interquartile range; range) test duration was 14 hours (10-16; 3-36) in patients with DI and 18 hours (14-24; 7-48) in patients with PP (*P*=0.011). Of those diagnosed with PP, 22 (26%) did not reach urinary specific gravity >1.020 nor urine osmolality >800 mOsm/L. Urine AVP did not overlap between patients with PP and patients with central DI. CONCLUSIONS: The short WDT is of limited value in the diagnostic work-up of polydipsia and polyuria and a partial DI may have been missed in every fourth patient diagnosed with PP. Urinary AVP has excellent potential in discriminating PP from central DI.

Key words: Diabetes insipidus, Polydipsia, Polyuria, Primary polydipsia, Urinary vasopressin, Water deprivation test

INTRODUCTION

The polyuria-polydipsia syndrome is defined by chronic diuresis of abnormally large volumes (>30ml/kg/day) of non-concentrated urine accompanied by increased thirst and fluid intake.[1] There are three types of the polyuria-polydipsia syndrome. Central, or neurogenic, diabetes insipidus (DI) is caused by a failure to produce and secrete the antidiuretic hormone agrinine-vasopressin (AVP). Nephrogenic DI is caused by AVP insensitivity in the kidneys. In both

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central and nephrogenic DI thirst, and subsequently water intake, are increased in order to reduce the rising plasma osmolality. The third cause of the polydipsia-polyuria syndrome is called primary polydipsia (PP), to distinguish it from the secondary polydipsia that follows the water loss in DI. AVP secretion in PP tends to be reduced due to lower plasma osmolality, while urine output is increased in order to prevent overhydration.[2-4]

It is crucial to accurately determine the aetiology of the polyuria-polydipsia syndrome as each underlying entity requires different treatment. The cornerstone of the diagnostic algorithm is a water deprivation test (WDT), often followed by administration of synthetic AVP (desmopressin). This protocol, originally developed by Miller et al,[5] has been used with various modifications in order to assess urinary concentrating ability as a function of AVP activity. Often, a diagnostic attempt is made on the basis of the clinical setting in which DI occurred, as well as based on urinary concentrating ability after an overnight (short) water deprivation. Only a few clinical studies, on a small series of patients, have been published to validate its utility, as recently reviewed by Fenske and Allolio.[6]

Direct evaluation of AVP activity by measuring plasma AVP has not been completely successful in exploring the polyuria-polydipsia syndrome because of the well-known pre-analytical instability of plasma AVP, as well as problems with the AVP assay.[6,7] Nevertheless, measurement of urinary AVP seemed to be a promising approach when studied by Dunger et al in children,[8] and later by Diederich[9] et al in adults, with the polyuria-polydipsia syndrome.

The aim of this study was to evaluate the clinical value of the short WDT, and an extended variant of the WDT, as well as to assess the diagnostic potential of urinary AVP in evaluating patients with the polyuria-polydipsia syndrome.

MATERIAL AND METHODS

Study design and participants

This was a retrospective, single-centre study based on results from 104 consecutive WDTs performed between 2004 and 2014. All patients had been referred to our department for evaluation of polyuria and/or polydipsia. Other causes of polyuria and polydipsia such as diabetes mellitus, hypercalcaemia and hypokalaemia had been ruled out prior to the WDT. Patients with the polyuria-polydipsia syndrome, referred for WDT, with urine osmolality >800 mOsm/L at baseline were excluded from the analysis (n=3).

All patients were followed for up to 12 years after the WDT with regard to treatment, i.e., whether they were on desmopressin treatment or not, and to any residual symptomatology related to the polyuria-polydipsia syndrome.

Water deprivation protocol

The same protocol was used during the entire study period and conducted by the same nurse. The patients were admitted to our ward the day before the test started. Patients with a reported urine volume <7 litres per 24 hours started the test at midnight, while those with a reported urine volume >7 litres started the test at 08:00 AM. Consumption of any liquids during the test was strictly prohibited and patients were not allowed to leave the ward. The test continued, without any duration limits, until one of the following criteria was met: i) More than 3% weight reduction, ii) Urinary specific gravity above 1.020 or, urine osmolality above 800 mOsm/L (considered to be diagnostic for PP), and iii) Intolerable adverse symptoms such as excessive thirst. Body weight, urine osmolality and urinary specific gravity were measured on every occasion the patient urinated throughout the test and not at predefined time points except for additional testing 8 hours after initiation of the WDT. Serum sodium and osmolality were measured at baseline, at 8 hours and at the end of the test. In the case of low urine osmolality <300 mOsm/Kg and a weight reduction of at least 3%, 4 µg of desmopressin (Minirin®; Ferring SA Holding Lausanne, CH. Switz.), was administered intravenously in order to differentiate between central and nephrogenic DI. In case of desmopressin administration, additional measurements of urine osmolality and urinary specific gravity were performed after one and three hours. At the end of the test, the diagnosis of PP, DI or partial DI was made with regard to the overall clinical setting of the patient.

In addition, urinary AVP was measured at the start and at the end of the WDT between 2008 and 2013 (n=54).

Biochemical analyses

Serum and urine osmolality were measured by depression of the freezing point method (2400 Osmometer, Fiske, Norwood, MA, USA) during the whole study period. The analytical range was 0-2000 mOsm/kg. The repeatability of the method was as follows: 0-400 mOsm/kg H_2O: ±3 mOsm/kg and 400-2000 mOsm/kg H_2O: ±0.75%, respectively.

Urinary specific gravity was assessed with Hydrometer ALLA360.100PMG, 1000-1060, 0.001g/ml, standardised at 20°C (ALLA France, Chemillé en Anjou - France). If the hydrometer reading was taken at a temperature other than the standard temperature for the hydrometer, the reading would be in error due to the change in volume of the hydrometer between the two temperatures. In that case, appropriate corrections were made according to the manufacturer's instructions.

Urinary AVP was measured as follows. Samples of 200 µL and calibrators of 100 µL were analysed in duplicates. After addition of 100 µL AVP antiserum (Baylis), final dilution 1/72 000 (until June 4, 2010), or a final dilution 1/80 000 (from June 15, 2010) in assay buffer (0.35% bovine serum albumin in 0.05 M sodium phosphate buffer, pH 7.4), samples were incubated at 6°C for 24 hours. A second incubation in the same manner was performed after addition of 100 µL 125I-AVP [Radioactive ligand was synthesised using a modified chloramine-T method,[10] purified with RP-HPLC using a µ-Bondapak C18 column (3.9×300 mm, 125 Å, 10 µm, Waters Code no. 27324), diluted 1/10 in CH3CN and stored at -20°C until use] (diluted to 5000 cpm 10% in assay buffer). Free and bound tracer were separated using 100 µL anti-rabbit IgG (AA-Sac1 from IDS), incubation at room temperature for 30 min, addition of 1 mL deionised water and centrifugation (2500×g, 21°C, 5 min). The supernatant was discarded and the precipitate measured in a gamma counter [automatic gamma counter, Wizard 1470, counting efficiency 75%, connected with an immunoassay software programme (MultiCalc Advanced, Wallac Oy, Finland)]. Mean coefficients of variation were 16% (range 13–18%) for internal control samples with a concentration of 0.4 pmol/L, 17% (range 15–20%) for 0.8 pmol/L and 17% (range 15–19%) for 2.2 pmol/L.

Ethical considerations

The study was approved by the Ethics Committee at the University of Gothenburg. The study was conducted according to the Declaration of Helsinki.

Statistical methods

All statistical analyses were performed with SPSS, version 22.0 for Windows. Data are presented as median [interquartile range (IQR)] for continuous variables and percentages for categorical variables. Regarding urinary AVP concentrations and duration of the WDT in the PP and DI group, respectively, range is also given. For comparison between groups, DI versus PP, the Mann-Whitney U-test was used for continuous variables and the χ^2 test was used for proportions. The significance level was set at 0.05 (two-sided p-value). Receiver operating characteristic (ROC) analysis of urine osmolality was performed in order to identify the cut-offs that give the best sensitivity in relation to specificity.

RESULTS

Subjects characteristics

The cohort consisted of 104 consecutive patients, of whom 64 (62%) were women. The median (IQR) age was 43 (30-58) years. The median reported fluid intake upon referral was 5 (4-6) litres per day (Table 1).

There was no difference in the reported fluid intake prior to the WDT between patients later diagnosed with DI as compared to patients later diagnosed with PP (Table 1; Figure 1). In contrast, serum osmolality and serum sodium levels were higher, whereas urine osmolality and urinary specific gravity were lower in patients with DI compared to those with PP.

Water deprivation test, DI versus PP

Of 104 patients, 21 (20%) were diagnosed with DI and 83 (80%) with PP. Seventeen patients had central DI in whom the most common cause was iatrogenic DI following transsphenoidal pituitary surgery (n=5), or other pituitary lesions [hypophysitis (n=2), Langerhans histiocytosis (n=2), Wegener's

granulomatosis (n=1), posttraumatic (n=1), unknown (n=1) and idiopathic DI (n=5). Four patients had nephrogenic DI, one due to treatment with lithium.

The median (IQR; range) time to termination of the test was 14 hours (10-16; 3-36) in patients with DI and 18 hours (14-24; 7-48) in patients with PP (*P*=0.010; Table 2 and Figure 2). Of those diagnosed with PP, 22 (26%) did not reach either urinary specific gravity above 1.020 or urine osmolality above 800 mOsm/L. Only 9 patients met the criteria for

Table 1. Baseline characteristics versus diagnostic outcome according to the water deprivation test in the study population.

	All n=104	Diabetes Insipidus n=21	Primary Polydipsia n=83	*P* DI versus PP
Sex, F/M	64/40	11/10	56/27	0.19
Age, years	43 (30-59)	53 (41-66)	40 (29-51)	**0.006**
Reported fluid intake, L/day	5.0 (4.0-6.0)	4.8 (4.0-8.8)	5.0 (4.0-6.0)	0.71
Serum osmolality at WDT start, mOsm/Kg	295 (290-298)	298 (297-304)	295 (290-298)	**0.002**
Serum sodium at WDT start, mmol/L	141 (140-144)	144 (143-146)	141 (140-143)	**<0.001**
Urine osmolality at WDT start, mOsm/kg	338 (170-491)	171 (110-369)	333 (171-476)	**0.010**
Urinary specific gravity at WDT start, g/ml	1.003 (1.000-1.008)	1.001 (1.000-1.004)	1.005 (1.001-1.009)	**0.004**

DI: Diabetes insipidus; PP: Primary polydipsia; WDT: Water deprivation test.
All continuous variables are presented as median (interquartile range). Significant *P*-values are shown in bold.

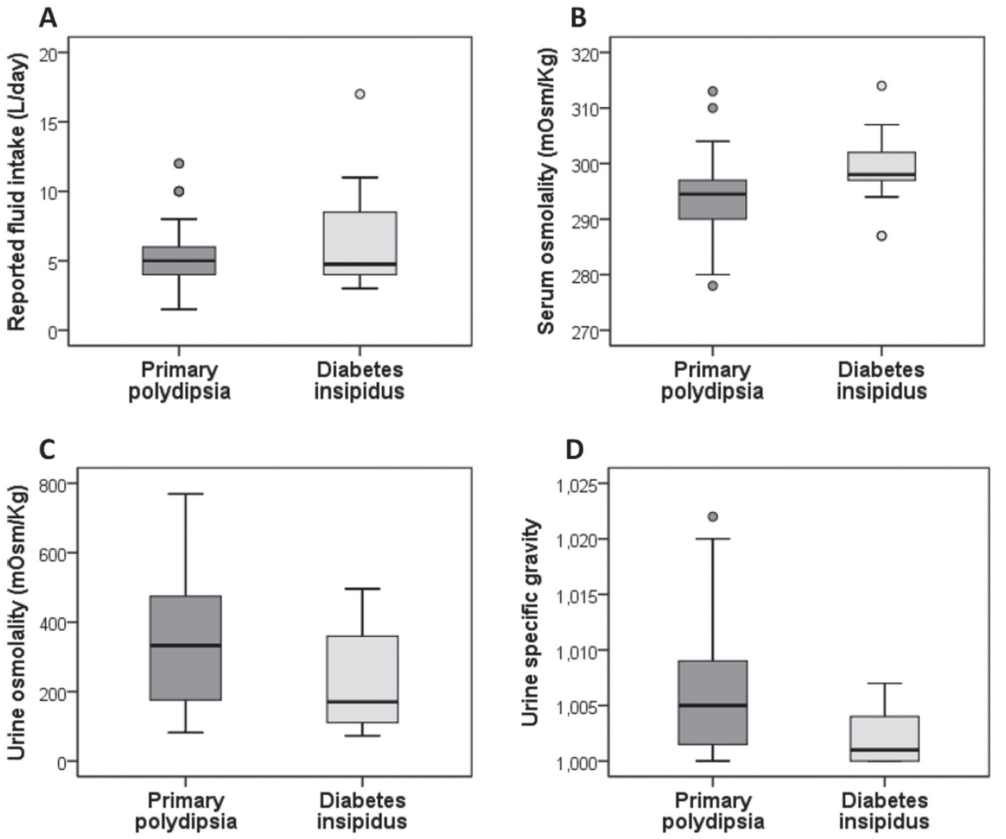

Figure 1. Box plots (median, interquartile range, 5th and 95th percentile) showing: A) Reported fluid intake prior to the WDT, B) Serum osmolality, C) Urine osmolality and, D) Urinary specific gravity, at baseline in patients diagnosed with diabetes insipidus and primary polydipsia.

termination of the test within 8 hours, of whom 4 (24%) were diagnosed with DI and 5 (6%) with PP (Table 2).

The median weight loss was 3.0% (2.1-3.4) in patients with DI and 2.1% (1.3-3.0) in patients with PP (Table 2). At the end of the WDT, serum osmolality,

Table 2. Results of the water deprivation test in the study population.

	All n=104	Diabetes Insipidus n=21	Primary Polydipsia n=83	*P* DI versus PP
Duration of WDT, hours	16.5 (13.5-23.8)	14.0 (10.0-16.3)	18 (13.5-24.0)	**0.010**
Patients who met criteria within 8 hours, n (%)	9 (9)	4 (19)	5 (6)	0.058
Maximum weight loss, %	2.3 (1.4-3.0)	3.0 (2.1-3.4)	2.1 (1.3-3.0)	**0.017**
Serum osmolality at WDT end, mOsm/Kg	198 (193-302)	305 (301-311)	297 (292-300)	**<0.001**
Maximum urine osmolality, mOsm/kg	744 (595-850)	286 (158-462)	802 (704-886)	**<0.001**
Maximum urinary specific gravity, g/ml	1.020 (1.012-1.021)	1.003 (1.000-1.010)	1.020 (1.017-1.021)	**<0.001**
Serum Sodium at WDT end, mmol/L	142 (140-144)	146 (144-151)	141 (140-144)	**<0.001**
Urinary volume output at WDT, end, mL/hour	30 (10-40)	70 (40-100)	30 (10-40)	**<0.001**

DI: Diabetes insipidus; PP: Primary polydipsia; WDT: Water deprivation test.
All continuous variables are presented as median (interquartile range). Significant P-values are shown in bold.

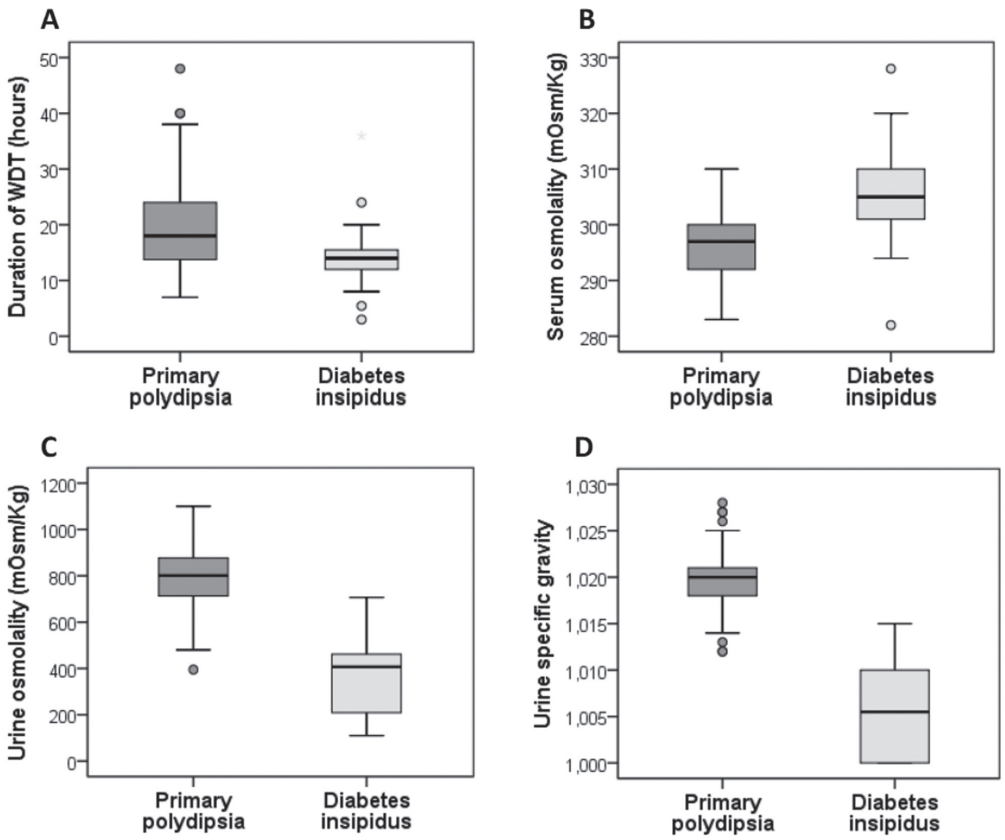

Figure 2. Box plots (median, interquartile range, 5th and 95th percentile) showing: A) Duration of the water deprivation test (WDT), B) Serum osmolality, C) Urine osmolality and, D) Urinary specific gravity, at the end of WDT in patients diagnosed with diabetes insipidus and primary polydipsia.

serum sodium and urine output volume were higher in patients with DI than in those with PP, whereas urine osmolality and urinary specific gravity were lower.

ROC analysis was performed for calculation of the cut-off values of urine osmolality that best differentiate DI from PP. A cut-off level of 600 mOsm/kg gave the best predictive value for DI diagnosis [area 0.96, (95% CI 0.93-1.0); P <0.001] with sensitivity and specificity of 90%, respectively.

All patients with PP had urine osmolality >400 mOsm/kg, corresponding to 100% sensitivity and 47% specificity (i.e., every other patient with urine osmolality >400 mOsm/kg had partial DI). None of the patients with DI had urine osmolality >710 mOsm/kg. The cut-off of 710 mOsm/kg corresponded to 100% sensitivity and 76% specificity for DI (i.e., every fourth patient with urine osmolality <710 mOsm/kg had PP).

When the cut-off value was set even higher at 800 mOsm/kg the specificity was 100% for excluding DI (i.e., only patients with PP had urinary concentrating capacity greater than 800 mOsm/kg), but the sensitivity in the ROC analysis was only 51% (i.e., up to every other patient with urinary concentrating capacity of less than 800 mOsm/kg could have had PP).

Urinary AVP at the start and the end of WDT

Urinary AVP was analysed in 54 patients: 41 with PP, 10 with central DI and 3 with nephrogenic DI. Patients with central DI had lower urinary AVP than patients with PP, both at the start and at the end of the WDT (Figure 3). In fact, the urinary AVP at the end of the WDT could discriminate central DI [median 2 (0.7-3.4); range 1-5 pmol/L] from PP [median 22 (13-40); 6-122 pmol/L] in 100% of the cases (Figure 3). The three patients with nephrogenic DI had urinary AVP concentrations comparable with patients with PP (data not shown).

Follow-up of the clinical diagnosis

The median follow-up time was 3 (5-7) years. All patients diagnosed with DI (n=21) were still on desmopressin treatment by the time of follow-up. Of 83 patients with PP, 3 (4%) reported residual symptoms and/or had started treatment with desmopressin.

DISCUSSION

This study, the largest series on WDTs performed at a single study centre, demonstrates the limited value of the short overnight test. Specifically, only 20% of the patients with DI met the diagnostic criteria within eight hours. For the vast majority it took, roughly, at least double the time. Similarly, the diagnosis of PP was made within the same time frame in only 6% of the patients. Furthermore, 26% of the patients who received the diagnosis of PP did not reach the cut-off of 800 mOsm/kg in urine osmolality, despite an unlimited duration of the WDT, for up to 48 hours

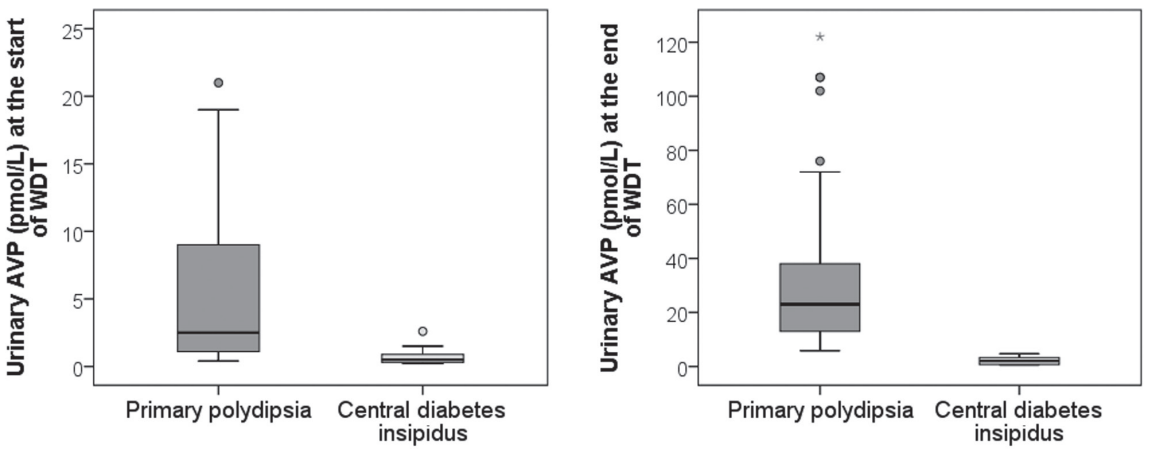

Figure 3. Box plots (median, interquartile range, 5th and 95th percentile) of urinary arginine-vasopressin (AVP) concentration at the start (left) and at the end (right) of the water deprivation test (WDT) in patients with primary polydipsia and central diabetes insipidus, respectively.

in one case. In other words, every fourth patient in the cohort, with possible partial DI, may have been misdiagnosed with PP.

The polyuria-polydipsia syndrome still remains a diagnostic challenge. Even though considerable efforts have been made to develop new methods to improve the accuracy of the diagnostic work-up, for instance measurements of direct AVP or copeptin, the WDT remains the gold standard.[6] Although the test has been used for decades, convincing cut-off levels that would add to the test's ability to differentiate between different aetiologies of the polyuria-polydipsia syndrome, in particular milder forms of DI and PP, are still lacking. The majority of the few studies available to date have evaluated the urine concentrating capacity after the administration of hypertonic solutions and not the most widely used WDT.[11-13]

Very few studies have validated the clinical utility of the WDT. In a study by Fenske et al, similar criteria were used as in the current study (with the exception that the duration of the test was limited to 16 hours), aiming at diagnosing DI or PP in 50 patients with the polyuria-polydipsia syndrome.[14] Without taking AVP measurements into account, the authors reported an overall correct diagnosis in 70% of the participants and in only 41% of patients with PP. The reference diagnosis was based on clinical information and treatment response. In a recently published study by de Fost et al, the diagnostic performance of WDT was evaluated prospectively in 40 patients with polyuria, followed for up to 18 years after the WDT had been performed.[15] The authors achieved a sensitivity of 100% in diagnosing DI when lowering the urine osmolality cut-off to 680 mOsm/kg compared to 96% using the widely accepted limit of 800 mOsm/kg. Similarly, in our study a reduced cut-off in urine osmolality at 710 mOsm/kg guaranteed 100% sensitivity in diagnosing DI.

It is obvious that the real challenge in clinical praxis is to discriminate PP from partial DI and the present study confirms the pitfalls in the currently recommended work-up algorithms. In fact, none of the variables analysed with the WDT can be used to perfectly discriminate between the two disorders. In agreement with Fost et al,[15] lowering the threshold of urine osmolality to 710 mOsm/kg safely rules out DI. However, it must be kept in mind that one fourth of patients with PP may still have urine osmolality below the cut-off of 710 mOsm/kg and can therefore not be distinguished from patients with DI.

On the other hand, in our cohort urinary AVP showed a remarkable diagnostic capacity to discriminate PP from central DI, including patients with partial DI. Despite the fact that earlier studies showed a promising performance of urinary AVP in both polyuric disorders,[16] as in normal subjects,[17] by testing it in both children[8,18] and adults,[9] this variable has not been integrated into clinical practice. As previously demonstrated, contrary to plasma AVP, urinary AVP is more stable[19] and exists in much higher concentrations than in plasma[8,17,16] so that even less-sensitive RIAs are equally adequate to detect it.[9] Thus, our results are in full agreement with those of Diederich et al[9] and the authors of the current report suggest taking the measurement of urinary AVP into account when assessing the various polyuric states.

The underlying mechanisms of the various disorders behind the polyuria-polydipsia syndrome contribute in the overlapping phenotypes in terms of symptoms and biochemical response to the WDT, thus making the interpretation of the results demanding. Patients with partial DI have still a considerable capacity to produce AVP under severe dehydration, whereas patients with acquired nephrogenic DI can have incomplete resistance to AVP.[1] Furthermore, patients with partial DI may present with normal urine concentrations, especially those with decreased glomerular filtration rate.[3,20] On the other hand, the chronic water diuresis in patients with PP may result in a "washout" of the renal medullar concentrating gradient and a down-regulation of the expressed aquaporin 2 channels and can, in this way, decrease the urinary concentration capacity.[21-23]

The major strength of the present report is the large number of WDTs included, all performed by the same experienced nurse at a single centre, using the same protocol throughout the study period. Moreover, the spectrum of the different underlying diagnoses was fairly wide, providing a clear patient-oriented

picture of the efficacy of the WDT. The limitations of the study are the retrospective design and that the follow-up of the clinical diagnosis was limited to reviewing the medical journals available in our region. Unfortunately, not many cases with central DI were included in the present cohort since the vast majority of central DI diagnoses in our clinic were made in the clinical setting of postoperative pituitary surgery or known hypothalamic-pituitary lesion. In addition, AVP or copeptin concentrations in serum were not taken into account. Nevertheless, the main aim of the study was to evaluate the diagnostic value of the WDT in terms of urine concentrating capacity and urine AVP, and in that regard no additional biochemical tests were warranted.

In conclusion, the short WDT is of limited value in the diagnostic work-up of patients with the polyuria-polydipsia syndrome when the evaluation is based solely on urinary osmolality. Despite the unlimited test duration for up to 48 hours, a partial DI may have been missed in every fourth patient. Urinary AVP had excellent diagnostic power in fully discriminating PP from all forms of central DI and could be useful in the diagnostic work-up of the polyuria-polydipsia syndrome. Larger studies are however warranted in order to further evaluate the clinical relevance of urinary AVP.

DECLARATION OF INTEREST

None of the authors has any conflict of interest.

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AUTHOR CONTRIBUTIONS

PT and OR performed the patient selection process and data collection. All authors contributed to the design of the study as well as to data interpretation and analysis, and in writing and revising the report. All authors are responsible for the integrity of the data and accuracy of the analysis, and all approved the final report.

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Case report

Medullary thyroid cancer, leukemia, mesothelioma and meningioma associated with germline *APC* and *RASAL1* variants: a new syndrome?

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ABSTRACT

Medullary thyroid carcinoma (MTC) is a neuroendocrine tumor hereditary in 35% of cases. The most common syndromic form is in the context of the multiple endocrine neoplasia type 2 (MEN 2) syndromes in association with other tumors and due to germline *RET* mutations. We describe a 57-year-old female patient diagnosed with sporadic MTC. The patient had a history of other neoplasias, such as acute myeloid leukemia, for which she had received chemotherapy, and two other solid tumors, peritoneal mesothelioma and meningioma. Genetic analyses were carried out including whole exome and Sanger sequencing (WES and SS) and loss-of-heterozygosity (LOH) testing for the respective loci. Immunohistochemistry (IHC) was used for the detection of proteins of interest. WES showed two germline variants in the *APC* and *RASAL1* genes confirmed by SS. In MTC tissue only there was a *RET* variant identified by SS; germline studies did not show any *RET* sequence changes. The pattern of tumors in this patient is unusual for either one of the *APC*- or *RASAL1*-associated neoplasms and her non-MEN 2-associated MTC contained a RET variant like other sporadic MTCs. As in other patients with more than one genetic variant predisposing to tumors, it is possible that this case represents a unique association.

Key words: Acute myeloid leukemia, Medullar thyroid carcinoma, Meningioma, MEN2, Peritoneal mesothelium, RET

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INTRODUCTION

Medullary thyroid carcinoma (MTC), a neuroendocrine tumor arising from parafollicular C-cells of the thyroid gland, accounts for 5-10% of all thyroid cancers, with a 1-2% incidence in nodular thyroid

disorders.[1] MTCs may be either sporadic or hereditary. Hereditary tumors are associated with multiple endocrine neoplasia (MEN) 2 syndrome: MEN2A, MEN2B and familial MTC (FMTC).[2] Sporadic forms account for 75% of cases, while MEN2A accounts for a little more than 34% of all hereditary MTCs, with a high (90%)[3,4] penetrance.[5]

MEN2A and MEN2B syndromes have distinctive phenotypes and autosomal dominant inheritance and are due to *RET* proto-oncogene genetic alterations.[3-5] The most common abnormalities that can occur in MEN2A patients are pheochromocytomas (in 40-60% of cases) and hyperparathyroidism (in 10-30% of cases).[6,7] MEN2B subjects have no observable parathyroid abnormalities; however, 40-60% of cases develop pheochromocytomas.[4] The presence of cutaneous lichen amyloidosis, pruritic plaques located on the upper back, is also reported in some kindreds with MEN2A or FMTC,[7] as well as Hirschsprung's disease in some MEN2A cases.[8] FMTC is also an autosomal dominant condition due to genetic alterations in the *RET* proto-oncogene. It has the least aggressive features[9] and is the most common form of hereditary MTC (57.6%).[4]

We present a patient with multiple endocrine and non-endocrine neoplasias— including MTC—which are not correlated and cannot be included in any of the known clinical syndromes associated with MTC. Genetic analyses revealed the presence of two *'de novo'* germline mutations, one in the adenomatous polyposis coli *(APC)* gene and the other in the RAS Protein Activator Like 1 *(RASAL1*) gene as well as one somatic mutation in the *RET* gene, which however is not yet known to be related to the MTC.

CASE PRESENTATION

A 57-year-old female was followed for bilateral MTC diagnosed 4 years ago. At that time she was submitted to total thyroidectomy as well as lymph nodes excision (7/17 positive) of the left cervical compartments. Histology showed that the carcinoma was extended with diffuse infiltration of both thyroid lobes and the paratracheal fat tissue (stage, T3m-N1M0). Imaging had showed no distal metastases. Pre-operative serum calcitonin levels were high at 140 μU/ml baseline and 244 after stimulation with calcium, while levels post-operatively were 14 μU/ml with normal serum CEA levels.

She presented to the outpatient endocrinology clinic of our hospital for her follow-up. Her family medical history was non-contributory. Her personal medical history included acute myeloid leuchemia (AML) diagnosed at the age of 41 years old and treated with chemotherapy (she had received initially two cycles of idarubicine/cytarabine with complete regression—however, she developed Sweet syndrome—and two cycles of floudarabin and cytarabine); since September 2002 she is considered in remission. Her medical history was also marked by a surgical exeresis of a peritoneal mesothelium at the age of 43 years old and a meningioma of 2×1.6×1.7 cm diagnosed at the age of 53 years old for which she has constant follow-up. The patient was also known to suffer from polyglandular autoimmune syndrome type 3 (PASIIIc) including Hashimoto's thyroiditis, autoimmune gastritis, alopecia universalis and relapsing perichondritis of the ear, while recently rheumatoid arthritis was also diagnosed.

METHODS

Genetic screening for *RET* gene variant

A peripheral blood sample was drawn in EDTA for molecular investigations after informed consent was obtained from the patient. DNA was extracted from peripheral blood leucocytes according to commercially available protocols (QIAGEN, Valencia, CA, USA). Genomic DNA fragments encompassing regions in the following exons: 5, 8, 10, 11, 13, 14, 15, previously found to contain activating missense *RET* mutations related to MTC, were amplified by polymerase chain reaction (PCR) and analyzed by classical bidirectional Sanger sequencing using BigDye Terminator V3.0 (Applied Biosystems).

Given this result, DNA was then extracted from paraffin-embedded thyroid tissue and DNA fragments encompassing all exons of the *RET* gene were amplified by PCR, while direct sequencing of the purified fragment was performed using BigDye Terminator V3.0 (Applied Biosystems).

RESULTS

No mutation in the aforementioned exons of the *RET* gene was detected in the blood sample. Interestingly, in the MTC tissue the variant c.1546C>T (ENST00000355710.7, NM_020975) was detected (Figure 1). The c.1546C>T leads to the replacement of the non-polar hydrophobic amino acid proline by the polar hydrophilic serine (p.P516S). This mutation is not reported either in gnomAD or in 1000GP. Further genetic analyses of blood samples, including SNP microarrays (comparative genomic hybridization; CGH) and WES (whole exome sequencing), were also performed as part of our diagnostic work-up.

Genetic analyses with CGH and WES

CGH showed no abnormalities. However, WES revealed the following two 'novel' mutations. One is heterozygous (c.3307 A>T) in exon 16 of the *APC* gene (NM_000038.5), leading to the replacement of arginine (R), a positively charged hydrophilic amino acid, by the hydrophobic tryptophan (W) (p.R1103W). The second (c.1613G>A) is also a heterozygous mutation in exon 16 of the *RASAL1* gene (NM_001193520). The c.1613 G>A *RASAL1* mutation, which leads to the amino acid replacement of arginine (R) by histidine (H) (p.R538H), was already described in the gnomAD database, with a frequency of 0.0037%.

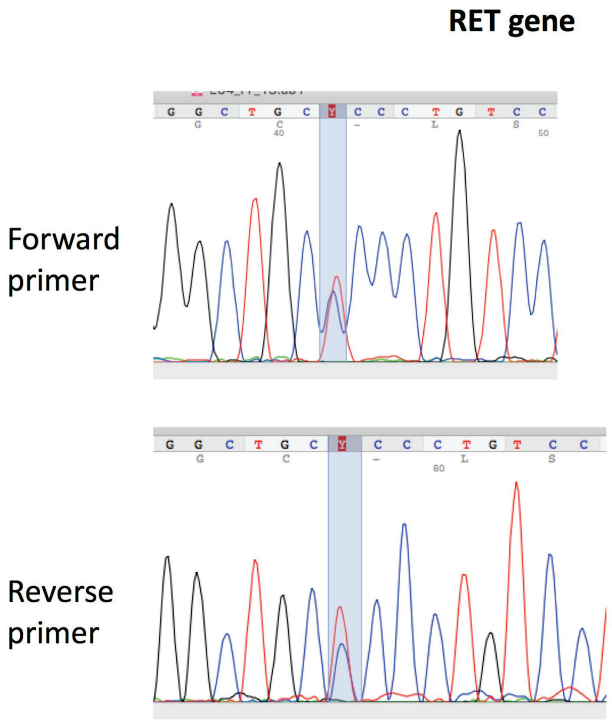

Figure 1. Electropherograms showing the analysis of the *RET* gene (Reference Sequence: NM_001193520) in the medullar thyroid carcinoma tissue showing the substitution of cytosine to thymine (c.1546C>T).

In silico analysis

Two sites of prediction of the functional effects of gene polymorphism were used: Polyphen (http://genetics.bwh.harvard.edu/pph2/), and SIFT (http://sift.jcvi.org/).

In silico analysis of the c.3307 A>T *APC* mutation predicts that this variant is probably damaging to the protein structure/function. The *in silico* characterization of the c.1613G>A *RASAL1* mutation is inconsistence in its predictions as to whether or not the variant is damaging to the protein function/structure.

Immunochemistry

Paraffin-embedded tissues from MTC were stained for APC and *RASAL1* protein expression (Figures 2, 3). MTC tissue showed an intense and diffuse staining for APC protein, whereas staining for *RASAL1* was faintly positive.

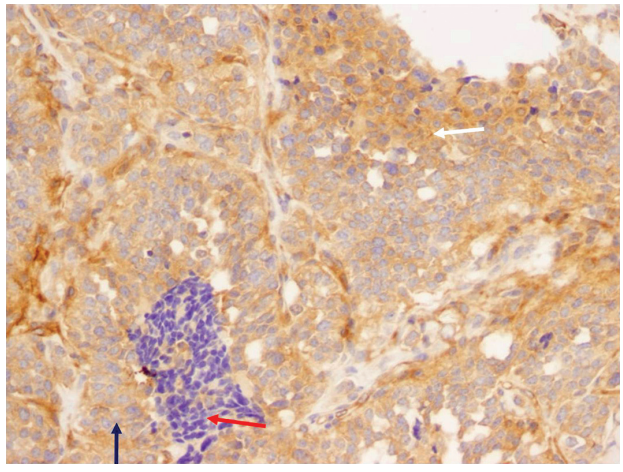

Figure 2. IHC analysis of paraffin-embedded MTC tissue revealed diffuse positive staining (20×). White arrow: Tumor cells with intense cytoplasmic immunoreactivity. Red arrow: Lymphocytic infiltration which is clearly negative (negative control). Blue arrow: Normal pattern for normal follicular cells: mostly negative—the nucleus is negative and the cytoplasm very weakly positive.

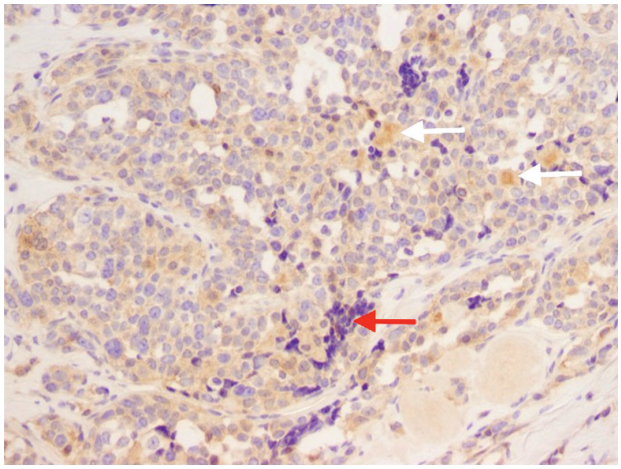

Figure 3. Faint staining of the *RASAL1* protein in the IHC analysis in the medullar thyroid carcinoma tissue (20×). Normally the *RASAL1* gene is very highly expressed in thyroid cells since the thyroid along with the brain and adrenal glands are the sites of highest expression. White arrow: Cluster of cells that are positive but that do not look like tumor cells. Red arrow: Lymph nodes: a negative control.

DISCUSSION

Herein, we report two novel germline mutations detected in the *APC* and *RASAL1* genes, identified through WES, in a patient presenting with multiple neoplasias such as MTC, AML, peritoneal mesothelioma and meningioma; we also found a somatic missense *RET* mutation in the MTC tissue.

Neither the germline heterozygous variants in the *APC* gene nor the *RASAL1* gene have been reported previously as pathogenic variants. Of note, the *APC* mutation has not been described as either pathogenic or benign in people of European or Africo-American origin participating in the NHLBI exome sequencing project, while the *RASAL1* mutation was already described in the gnomAD database, with a frequency of 0.0037%.

The *APC* gene is an oncosuppressive gene involved in the WNT signaling pathway. Approximately 25% of pathogenic variants in the *APC* gene occur *de novo*.[10] The p.R1103W variant is a non-conservative amino acid substitution and occurs in a position within the 15-amino acid, repeat β-catenin binding domain that is conserved across species. Pathogenic truncating variants in the *APC* gene are most commonly associated with autosomal dominant familial adenomatous polyposis (FAP) characterized by early-onset presentation of hundreds of adenomatous colon and rectal polyps as well as duodenal, stomach, thyroid, pancreatic, brain and liver cancers.[10,11] Variable presentations of FAP include also Gardner syndrome (osteomas and soft tissue tumor) and Turcot syndrome (colonic polyposis and central nervous system tumors like medulloblastoma and malignant glioma).[12]

Rare somatic variants have been reported in individuals with hepatoblastoma and gastric cancer (MIM 611731). Interestingly, APC staining was found strongly positive in the MTC tissue with characteristic cytoplasmic localization; it could be hypothesized that the presence of this mutation could abolish the entrance of APC into the nucleus, keeping it mainly in the cytoplasm. The *RASAL1* gene encodes the RAS GTP-ase which serves as an inhibitory regulator of the Ras-cyclic AMK pathway that is expressed in a limited number of tissues, with the highest expression in the adult brain, thyroid gland and adrenal gland (MIM 604118).[13]

Follicular variant papillary thyroid cancer presents a higher frequency among individuals with *RASAL1* variants compared to those without.[13] Therefore, germline *RASAL1* variants may be relatively frequent in patients with apparently sporadic thyroid carcinoma with follicular features.[13] The R538H variant is a conservative amino acid substitution which is not likely to impact secondary protein structures as these residues share similar properties. The substitution occurs at a position that it is not conserved among species. *In silico* analysis for this variant was not clear. Thus this variant was interpreted as being of uncertain significance and could be related to the thyroid cancer reported in this patient. *RASAL1* staining in the tissue was less intense compared to APC.

The somatic variant in the *RET* detected in the MTC tissue has not been previously described. The co-existence of multiple neoplasias without common histological and embryological origin in this patient cannot be included in a specific genetic pattern or clinical syndrome according to the existing data in the literature. A secondary cause cannot be excluded in this patient already diagnosed with AML and treated with chemotherapy as it is known that prior exposure to chemotherapy can result in various types of somatic

mutation or even germ mutation, thus predisposing to a second neoplasm.[14-16] Interestingly, there are some rare cases reporting occurrence of papillary thyroid cancer and meningioma in patients treated for acute lymphocytic leukemia or some years after bone marrow transplantation, especially during childhood.[17,18]

CONCLUSION

Patients with 'mixed or atypical' phenotypes often present a diagnostic challenge. Genetic counseling in these cases is difficult because there is no specific genetic pattern. Here we report the case of a patient with multiple neoplasias including MTC which are not correlated and cannot be included in any known clinical syndrome. Genetic analyses showed that the patient presents two new germline mutations '*de novo'*, one in the adenomatous polyposis coli (*APC)* gene and the other in the RAS Protein Activator Like 1 gene as well as one somatic mutation in the *RET* gene, which however have never before been identified in MTC. The mutations in the *APC* and *RASAL1* genes are probably involved in thyroid cancer development, along with the somatic mutation in the *RET* gene. In addition, the mesothelioma and meningioma are likely related to mutations in the *APC* gene. Both germline mutations were characterized as variants of uncertain significance and they have not been previously described as pathogenic.

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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PATIENT CONSENT

Written informed consent was obtained from the patient.

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Commentary

Sarcopenia: From definition to treatment

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INTRODUCTION

Over the last few decades, worldwide average life expectancy has grown significantly and therefore the percentage of the elderly has correspondingly increased. As a result, clinical medicine has focused much interest on the latter age group and their health problems. During the 20th century, the typical elderly patient was a person with an acute or chronic disease but typically without any major disability. In contrast, the 21st century older patient's profile is characterized by considerable comorbidity, including a great number of chronic conditions with frequent acute episodes, all of which has an adverse effect on functionality. Accordingly, while the health system once focused on treating exclusively the disease, it nowadays needs to give greater attention to the rehabilitation of disability.[1]

Key words: DXA, Drugs, Nutrition, Physical activity, Rehabilitation, Sarcopenia

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Age-related muscle loss is one of the main characteristics of ageing. It appears more often in physically inactive people, although it also affects those who have been active throughout their lifetime,[1] this indicating that while the modern sedentary lifestyle is contributive to this phenomenon, it is not the only causative factor. Numerous changes take place as we grow old: apart from the aforementioned increasing tendency towards a sedentary lifestyle, there are changes in hormone levels and in the body's protein demands as well as the unavoidable degeneration (death) of the motor neurons.[2]

SEEKING A DEFINITION OF SARCOPENIA

Sarcopenia, an age-related loss of muscle mass and power, has recently been recognized as a disease. It has been included in the 10th edition of the ICD-10 (the International Statistical Classification of Diseases and Related Health Problems), in the categories "muscle wasting and atrophy not elsewhere classified" (code M62.5) and "disorder of muscle, unspecified" (M62.9), and since September 2016 it has been assigned code M62.84. The latter has constituted an important step towards the recognition of sarcopenia as a disease and, what is more, it is expected to increase the availability of diagnostic methods (diagnostic lab tests and trials) and to

motivate the pharmaceutical companies to develop appropriate medical treatment.[3]

Despite its recognition as a disease, sarcopenia does not as yet constitute adequate indication for therapeutic intervention; thus, interest is focused on the circumstances and the condition under which such organizations as the FDA (Food and Drug Administration) could consider sarcopenia as an indication for treatment.[4]

Irwin Rosenberg, who coined the term 'sarcopenia' in 1989, meticulously investigated the disorder, particularly stressing the importance of loss of muscle mass in the process of increasing disability. He defined sarcopenia as the observed age-related decrease in muscle mass and the decrease of muscle strength and functionality that concurrently takes place. The definition has been steadily improved since 1989 as well as since the first Sarcopenia Workshop which took place in 1994 organized by the National Ageing Research Institute. Today, the definition of sarcopenia considers both the loss of muscle function (muscle power) and the loss of muscle quality (muscle strength/muscle mass unit), in other words, the increasing inability of muscle tissue to generate force in addition to the loss of muscle protein. It has not yet been clarified whether the loss of muscle function in the elderly (difficulties in e.g. rising from a chair, avoiding falls, walking in the street, carrying weights) is mainly the result of a decrease in muscle mass or a deterioration in the quality of muscle tissue.[5]

Generally speaking, low muscle mass is common among the elderly and numerous studies have been designed to determine the severity of the problem. Certain studies have examined the frequency of the disease among the population (male and female) of the USA (NMEHS, NHANES III, CHS, ABC REP) and others populations of Europe and Asia (INCHIANTI, EPIDOS, LASA, CHINESE). The results have varied, with the estimated prevalence ranging from 10% to 50% depending on the study and a discordance existing regarding the gender more at risk. Given that until recently an articulate mutual definition of sarcopenia had not been found and that the criteria of participation and elimination are likely to have been different in each study brings into question the credibility of the results and limits the possibility of generalization. In one reliable study, the prevalence of sarcopenia was dramatically increased along with age, from 4% in men and 3% in women aged 70-75 years old to 16% and 13%, respectively, in persons aged 85 or more.[6] It is nevertheless well established that with advancing age, muscle mass and power deteriorate and their decline below a certain level (cutoff) leads to disability. Certainly, the disability cutoff is affected by other factors.[7]

Moreover, it has been observed that patients suffering from both sarcopenia and osteoporosis were at a greater risk of falls, therefore the incidence of fractures in this cohort was higher.[8] It is thus evident that the treatment of sarcopenia will considerably improve patients' wellbeing, especially if they are dealing with other diseases related to senility.

NHANES III (the National Health and Nutrition Examination Survey) has recorded the increasing frequency of sarcopenia with advancing age. However, in this study the description of sarcopenia was defined with no criteria other than muscle mass, whilst there is no information considering cachexia (due to other diseases), starvation (nutritional deficiency) and frailty (functional inability).[9]

As far as the definition of sarcopenia is concerned, it is usually complicated by the fact that there is an overlap between the diseases/conditions cachexia, sarcopenia, starvation and frailty. This conceptual confusion, found even among specialists, is due to the fact that all the abovementioned lead to muscle loss and are frequently related to each other. It is crucial that a clarification be made in clinical practice as different clinical and therapeutic treatment is required in each case.[10]

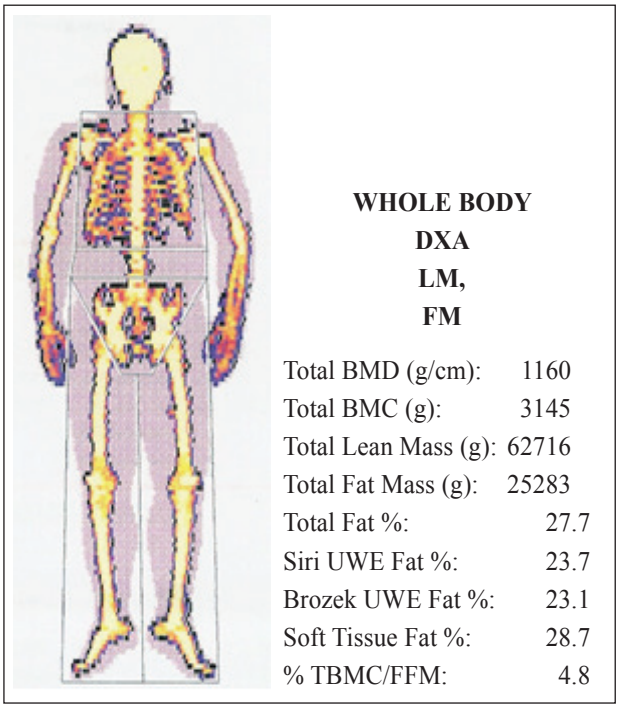
The formulation of an accurate definition will enable achievement of a more accurate diagnosis and of better designed clinical studies so that a conclusion may be drawn regarding the adequacy of the present therapeutic interventions. Just as for osteoporosis, a very frequent problem among elders, there are a number of medical options, there is likewise a need for drugs and lifestyle changes for the improvement of sarcopenia and of the functional ability of the elderly. Moreover, there are the financial costs that must be considered (incurred due to mobility impairment,

the toxicity of some drug combinations, infections during hospitalization), the total exceeding the one of the osteoporotic fractures i.e. 18.5 versus 16.3 billion dollars, respectively.[11]

Because of the lack of a consistent operational definition, different parameters have been assessed and a variety of testing methods have been used by the many investigations into sarcopenia. More specifically, Baumgartner et al defined the presence or absence of sarcopenia according to muscle mass (ASM-appendicular skeletal muscle mass) measured via the DXA (dual energy X-ray absorptiometry) method (Figure 1). Patients were considered as suffering from sarcopenia whose ratio ASM/height^2 was less than 2 standard deviation (SD) compared to the expected normal value for the average young adult. The DXA scan has over the years provided an easy diagnostic method: however, it is not available in all health systems and, moreover, it does not evaluate functional ability.[12]

The European Working Group on Sarcopenia in Older People (EWGSOP) and the International Sarcopenia Consensus Conference Working Group (ISCCWG) have pointed to the growing prevalence of the disease and have reviewed the scientific facts and the literature with regard to therapeutic intervention.[13] EWGSOP defined sarcopenia as the syndrome characterized by a progressive and, eventually, total loss of skeletal muscle mass and strength, this being additionally associated with the risk of bodily and mental impairment, poor quality of life and early mortality. There are two criteria for diagnosing sarcopenia: low muscle mass + low muscle strength and low physical performance (decreased functionality)[8] (Figure 2).

Using techniques such as DXA and BIA (Bioimpedance Analysis) and tests including grasping dynamometry and walking speed (gait speed test) or the SPPB test (Short Physical Performance Battery), the EWGSOP recommended that diagnosis be based on low muscle mass + low muscle function (strength or performance). Patients with less than 2 standard deviation (SD) compared to the expected normal value for the average young adult had sarcopenia. The main advantage of this definition is that it additionally provided information about muscle functionality. The EWGSOP categorized sarcopenia, according to the cause, into primary (or age-related) and secondary (when one or more non-age-related causes are evident) sarcopenia. Secondary sarcopenia has been further subdivided into sarcopenia related to: physical inactivity (e.g. after prolonged bed rest, low everyday/habitual physical activity, sedentary



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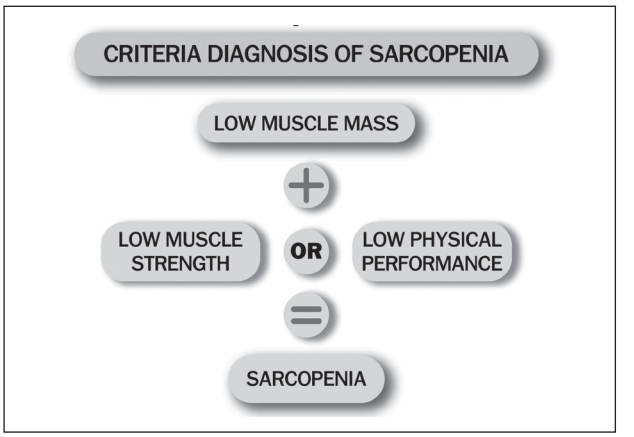

Figure 2. The European Working Group on Sarcopenia in Older People (EWGSOP).

lifestyle), a particular disease (e.g. advanced organ failure, inflammatory diseases, malignancy, endocrinopathy) and lastly, nutrition (less-than-optimal diet, malabsorption, gastrointestinal disorders, drug-induced anorexia) (Figure 3).

The ISCCWG's definition of sarcopenia was based on muscle mass and physical performance. After a meeting in Rome, ISCCWG published a Consensus that sarcopenia should be evaluated in older adults with clinically observed declines in physical functioning, activities of daily living, strength and health status, and those experiencing recurrent falls, recent weight loss, hospitalization or chronic conditions associated with muscle loss.[14]

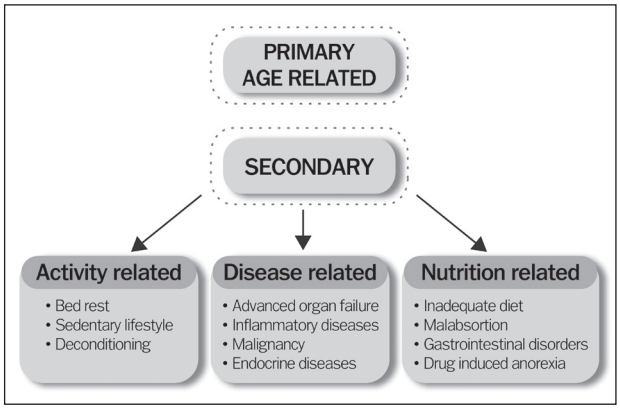

Figure 3. EWGSOP - Sarcopenia categories.

PATHOPHYSIOLOGY OF PRIMARY (AGE-RELATED) SARCOPENIA

The pathophysiology of sarcopenia in the elderly is complex with a plethora of internal and external procedures contributing to its development. As regards internal processes, the most important factors are the decrease of anabolic hormones (testosterone, estrogens, growth hormone, etc), the increased apoptotic activity of muscle fibers, the increase in pre-inflammatory cytokines (TNF-a, IL-6), oxidative stress due to accumulation of reactive oxygen radicals, the change in mitochondrial activity in muscle cells and the decrease in motor neurons. The external factors related to low functionality in the elderly include an insufficient intake of dietary protein resulting in loss of muscle mass and function, vitamin D deficiency, acute and chronic comorbidities leading to decreased physical activity and often to periods of prolonged immobilization and an age- and/or disease-related increased production of proinflammatory cytokines that induces proteolysis (cachexia).[15]

As a person ages changes take place in their protein metabolism since ageing affects systemic factors and hormones that are involved in the construction and deconstruction of proteins. For example, in elderly patients the observed decreased effectiveness of GH (growth hormone), IGF-1 (insulin-like growth factor-1) and insulin together with the concurrent increased activity of cytokines, catabolic hormone cortisol and myostatin adversely affect and inhibit protein biosynthesis. Myostatin is a hormone that acts on the myosatellite cells (precursors to skeletal muscle cells) and inhibits their growth and differentiation to mature muscle cells.[16]

The importance of muscle tissue for optimal functioning of the body is, from the biochemical point of view, enormous.[17] The muscle system is the body's largest storage depot for protein, which in such conditions as stress and malnutrition furnishes constant amino acid supplementation so that protein synthesis may be continued in other basic tissues. Since the majority of glucose is stored in skeletal muscles, decreased muscle mass plays a major role in the disordered glucose metabolism of patients with insulin intolerance and diabetes mellitus type II. Moreover, given that skeletal muscle is the highest consumer of energy (chemical energy), thus positively contributing to basal metabolic rate (BMR), muscle loss in the elderly is the main cause of age-related decreased BMR and energy expenditure. The possible connection between low muscle mass and functionality and pathological glucose tolerance was reported in a publication by Hedman et al which showed that the association between the muscle fiber synthesis and glucose sensitivity though important is not strong ($r=0.33$).[18] It is known that glucose transporter 4 (GLUT 4) plays a major role in regulating glucose homeostasis, with intracellular glucose transport being triggered by insulin, muscle contractions or exercise. Meanwhile, care must be exercised with regard to identifying the mechanisms that cause loss of strength in the elderly. First, elderly persons who lead a sedentary

lifestyle and at the same time have circulation and respiratory problems though also presenting "normal" mechanical muscle strength are probably suffering from a glucose muscle transport disorder. Second, there are thought to be patients who are being treated as diabetics but who are actually suffering from an undisclosed muscle disorder[17]

Old age comes with significant changes in muscle tissue architecture. There is a loss of motor units, due to the denervation of the fast twitch motor units, that include type II muscle fibers and, to a smaller degree, slow twitch motor units (with type I muscle fibers). After denervation, the muscle fibers can be recruited from the remaining motor units, at the same time changing their type, according to the muscle fiber type contained in the motor unit which recruits them. As a result, some denervated type II muscle fibers (which, when contracted, produce great power but for a short time) are turned into type I muscle fibers (which produce a smaller amount of power but last less time). Thus, with advancing age muscle tissue content in type I muscle fibers increases, while the number and mass of type II muscle fibers decreases (due to atrophy), which leads to a significant reduction in the maximum power that the muscle can produce. In elderly patients with sarcopenia, no recruitment takes place following muscle fiber denervation, which causes an even greater loss of motor muscle units, muscle atrophy and fraility.[16] Another change that comes with age is related to the elasticity of the muscle-tendon system or muscle-tendon unit. The tendon, as the weakest link in the chain, is the final regulator of all the region's strength connections (muscle-bones). The tendons have an essential role in body movement since they transmit force between bones and muscles. As a result, age-related modifications in movement are intimately bound up with the tendon's mechanical properties, which, together with the muscles, exert an integrated effect. The loss of tendon elasticity with advancing age thus induces a decrease of muscle contraction capacity.[16,19]

DIAGNOSIS OF SARCOPENIA

Regarding the diagnosis of sarcopenia, it must be specified, in accordance with the definition and using adequate measurements and tests, whether there is a loss of muscle mass, a decrease of muscle power or a disorder of functionality and physical performance.[8]

Determination of muscle mass can be made using anthropometric methods (height, weight and other body measurements), which are technically easy to carry out and low-cost but lack reliability. In addition, biological methods may be used (creatinine secretion, whole body potassium using radioisotope ^{40}K), which are more complex but more dependable. Lastly, imaging methods can be used (MRI, CT-scan) which, despite their high cost, the difficulty in performing them and the small exposure to radiation, provide valuable information about muscle quality. Analysis of body composition and determination of muscle mass percentage can be achieved using surface electrodes and via the bio-impedancemetry method, which is easily applied and low-cost, albeit not entirely reliable. DXA appears to be quite useful as it has a low cost, is easy to use and is also reliable. For the diagnosis of sarcopenia, measurement of appendicular muscle mass (ASM), i.e. of the skeletal muscle mass of the four limbs, needs to be taken[20] (Figure 4).

DXA systems provide a measurement of appendicular lean soft tissue (ALST), which is a fat and bone mineral-free tissue including muscle and other components such as skin, tendons and connective tissue. SM constitutes the largest fraction of ALST. Ap-

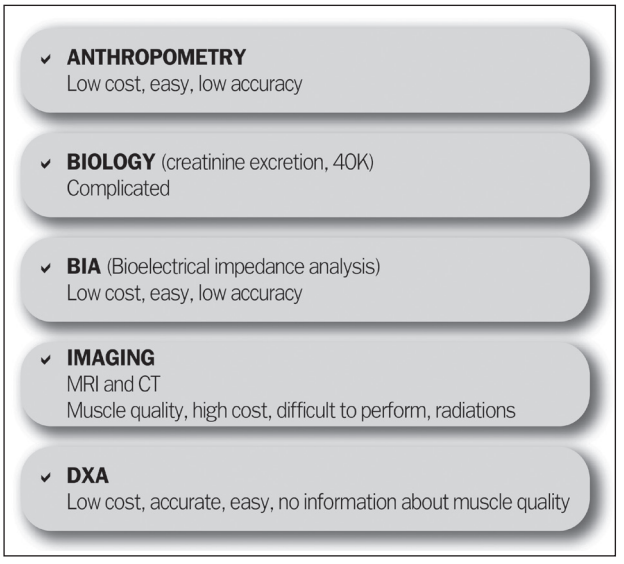

Figure 4. Muscle mass assessment tools.

pendicular skeletal muscle mass (ASM), as measured by DXA, is quite helpful in diagnosing sarcopenia.[21]

In the Rosetta study examining the frequency of sarcopenia in an elderly population in New Mexico the diagnosis of sarcopenia was based on the skeletal muscle mass index (SMI), which is the ASM to the height^2 ratio (SMI=ASM/height^2, counting units: kg/m^2). Indicatives of sarcopenia were considered to be the SMI values which were less than 2 standard deviations (SD) below the mean value of a young population's SMI. More specifically, the limit for men was 7.26 kg/m^2 and for women 5.45 kg/m^2. The strategy used to diagnose sarcopenia was similar to that used for osteoporosis. To set the diagnosis of osteoporosis, superficial bone density (gr/cm^2) measured by DXA (gold standard for the diagnosis of osteoporosis) is defined by the T-score, the number of standard deviations of the patient's value in comparison to mean bone density value of a normal young adult of the same gender and race. Thus, if the patient's T-score is less than 2.5 standard deviations from the reference value, the patient is suffering from osteoporosis. The 2.5 standard deviation cut-off was chosen based on the observed fracture risk (specific hip fracture risk). For a diagnosis of sarcopenia, muscle mass is measured and subsequently the T-score is calculated.[12]

For the purpose of determining the quantitative relationship between muscle mass and disability (decreased physical performance and functionality), Janssen et al studied 4,449 individuals who participated in the NHANES study during the period 1988-1994. Using the SMI as an indicator for the diagnosis of sarcopenia they came to the following conclusions: people with a SMI value of 8.51-10.75 in men and 5.75-6.75 in women were at medium risk for disability, whilst those with an even lower SMI (less than 8.50 in men and 5.75 in women) were at a greater risk, generally showing a higher risk of physical disability.[9]

However, they found an increased physical disability risk in women with very high SMI values. The increased physical disability risk in women with very high SMI values may have in part reflected the increased fat mass and obesity in these subjects. Fat mass is an independent predictor of physical disability and fat mass was considerably higher (39.2 kg vs. 28.1 kg) in women with very high SMI values ($\geq$9.00 kg/m^2) than in women with moderately high SMI values (6.75-8.99 kg/m^2).[9]

To conclude, using the SMI to define sarcopenia has some significant advantages, including that it is convenient, quick and easy to apply. However, there are a few serious disadvantages. These include the need to use DXA, which might not be available in every center, the definition of the threshold criteria which often differ, not taking into account the person's body weight and whether they are thin or obese (for example, thin people may have a low muscle mass without having any mobility limitations, while obese persons with generally a high muscle mass, but still lower in comparison to their body weight, may experience mobility limitations) and, finally, the fact that while focusing on muscle mass, a crucial parameter for the definition of sarcopenia, it does not take into consideration the strength that is produced per muscle mass unit and which differs greatly between the two genders and steadily declines with age.

On the other hand, it has been shown that apart from muscle mass (especially of the low limbs), muscle power independently defines the degree of motor impairment. Nevertheless, these two parameters are related, as a strong connection between lower limb muscle mass and muscle power has been found ($r=0.78$, $p<0.01$), while muscle mass is responsible for 60% of the total change in muscle power. Adequate muscle strength and power depend on multiple factors and thus additionally demand good functioning of the cerebral motor cortex, an intact spinal cord, normal conduction of electrical impulses, normal muscle mass and muscle tissue architecture, low density of myocytes in fatty acids and a normal stimulation-contraction junction.[22]

The next step in the diagnostic procedure is the use of tests that evaluate physical performance or muscle strength (possibly also power).[23] To assess physical performance, some tests are used such as the chair stand test (sitting-rising test), the walk test (walking test), the functional reach test (achievable functionality test) and the modified Romberg test. Another test to evaluate static and dynamic balance is the Timed Up and Go Test (TUG) during which the patient stands up from the chair, walks 3 meters, turns round, walks back to the chair and sits

down again. This test assesses only basic everyday moves, such as rising, walking, turning and sitting but not walking over an obstacle, which can cause slips and falls.

In general, some of the tests and measurements that can reveal limitations in functionality and physical activity caused by sarcopenia are the following: knee extension isometric torque, grasping power, lower limb muscle strength, 4- and 6-minute walking tests, the Short Physical Performance Battery (SPPB) that scores walking and balancing performance, the rising from a chair test and, lastly, the one-leg balance test.

The measurement of muscle power can be made with isometric methods, although these are static measurements, as they do not reproduce normal movement, or with isokinetic methods, which have the limitation of making the movement at specific angular velocities, which is a situation that is far from normal.

When measuring performance, the movements must be described by terms that include force, velocity and acceleration. Force (N) causes acceleration (a). Every movement is caused by the action of a force at some time, thus it should be measured as Power (W) (force x distance = work, work/time and force × velocity = power).[24]

Two tests, jumping mechanography and rising from a chair, measure the power during that activity. Jumping mechanography can be used in very frail people who are not able to get up from a chair. The evaluation of muscle quality is made with measurements that evaluate muscle mass and functionality and includes definition of muscle mass and assessment of functionality through a series of tests such as grip strength, knee extension power and muscle power. These tests, designed to identify patients with motor problems (and presenting some small differences in sensitivity and specificity), can be easily performed in the clinical setting, while they are also readily available and quite simple as they do not require any devices. Nevertheless, their validity is limited due to the fact that the normal threshold is not strictly defined and the patient's performance depends on such factors as motivation, will and pain (for example, a patient with osteoarthritis), and not only on muscle mass, power and strength.[25]

In order to complete a patient's investigation their biochemical profile needs to be assessed, including inflammatory markers (CRP, interleukin-6, TNF-a), clinical-pathological markers (hemoglobin, albumin, urine creatinine), hormone levels (testosterone, insulin-like growth factor-1, vitamin D).[26]

PREVENTION-TREATMENT-REHABILITATION: BASIC PRINCIPLES AND FUTURE TARGETS

Nutrition

One of the interventions for the prevention and treatment of sarcopenia is nutrition. Malnutrition is responsible for numerous health problems, one of which is the loss of muscle mass. Older adults often have reduced food intake and therefore are vulnerable to malnutrition.[27,28] Especially for someone who already suffers from sarcopenia, malnutrition can cause protein deficiency, further worsening the loss of muscle mass. An American survey showed that in the over 50 years age group, 32-41% of women and 22-38% of men consume less than the recommended daily protein intake.[29] Meanwhile, a 3-year study showed that protein intake maintained muscle mass in women and men aged 70-79. People with the highest daily dietary protein intake at the time of this study lost 40% less muscle mass than those with the lowest intake.[30] It has in addition been found that diets rich in acid-producing foods (such as meat and cereal grains) and poor in food that do not produce acid (fruit and vegetables) have a negative effect on muscle mass.[31] The above diet deficiencies in conjunction with the fact that the elderly tend to take in less calories in general can lead to severe protein deficits as well as deficiencies in other nutritive elements.

It has in general been shown that adequate food intake in elderly people protects them from sarcopenia, also that older adults are likely to need more protein per kilogram than young people in order to preserve adequate protein levels that support muscle mass[32,33] with about 1.0-1.2 gr/kg protein intake per day probably being optimal for this age group.

In conclusion, the maintenance of adequate protein intake is of crucial importance during the treatment of sarcopenia. For this purpose a number of nutritive and dietary supplements can be used.

There is some evidence suggesting that creatine supplements may help in muscle development in older adults who follow a training program with resistance exercise.[34,35] Maintaining the right vitamin D blood levels might also help preserve muscle power. In a study concerning clinical interventions in sarcopenia and a healthy appearance among elderly people in developed countries, a strong correlation was found between protein intake per meal and loss of muscle mass. Moreover, it has been noted that apart from an inadequate? dietary protein intake, certain other? adverse factors need to be taken into account: insulin resistance, cytokines, vitamin D deficiency and a sedentary lifestyle. All such data will provide a clearer understanding of the negative changes in body composition as age advances and thereby contribute to the designing of more effective therapeutic interventions.[35]

Physical Activity

The contribution of exercise, especially resistance exercise (or strengthening exercise), in the prevention of sarcopenia is of substantial importance. Resistance exercises positively impact the neuromuscular system, protein synthesis and the hormones, which cause sarcopenia when they function abnormally. Studies have shown that after a resistance exercise program, the triggering of the motor neurons and of protein synthesis (both essential to build muscle mass) increase, even in the elderly.[36,37] These changes indicate that the redevelopment of muscle mass is possible even in people of an advanced age.

More specifically, resistance exercise is considered to be a type of "strength" training increases maximum strength since persistent training is thought to enable an individual to progressively lift ever bigger weights. However, taking the physical laws into consideration, the issue is more complicated. Supposing that a 100kg object (total weight: 100kg × 9.81 m/s ≈1000 N) must be lifted in 1 min, the energy that must be produced will be 1000 N × 1m = 1000 J. If the above takes place in 1 sec, the result is an average power of 1000 j/1 sec = 1000 W, while if it takes place in 0.5 sec the average power is 2000 W (1000 j/0.5 sec= 2000 W). To conclude, from the viewpoint of mechanical physics, exercises using weights are intended for increasing power, not force.

Aerobic exercise also appears to help fight sarcopenia. This type of exercise has been shown to help increase protein synthesis, an important function for preservation of muscle mass and strength in the elderly.[37] Of note, though certainly physical activity improves and even prevents sarcopenia, this must be accompanied by good nutrition.

Drugs

There is an attempt to design medications to both prevent and treat sarcopenia and indeed numerous pharmaceutical drugs have been tested in various clinical trials.

Theoretically, the ideal drug should be able to achieve increase of muscle and bone tissue mass while simultaneously decreasing fatty tissue. A protein that is being investigated for its effect on muscle tissue mass is myostatin (GDF-8-growth differentiation factor-8).[38] Myostatin is produced by myocytes and acts on the autocrine function of muscle cells to inhibit their growth and differentiation, thereby inhibiting the synthesis of muscle tissue. Animals with mutations in the myostatin gene that lead to the inability to produce functioning myostatin and animals treated with substances that block the activity of this protein have significantly more muscle mass (for example, the Belgian Blue cow). Myostatin binds to the ARIIB receptor (activin receptor type IIB) and modifies the metabolic activity and homeostasis of the muscle via three intracellular pathways.[39] In patients with cachexia due to chronic disease, cancer or acquired immunodeficiency syndrome, increased myostatin levels have been found which is probably responsible for the low muscle mass in these individuals. It is generally known that myostatin levels are related not only to muscle mass but also to functional muscle parameters, such as power and force. Furthermore, myostatin is decreased during exercise and is related to the type of exercise (resistance exercise > endurance exercise), the intensity (light resistance does not modify it, while medium resistance does), as well as the person's potential (inactive individuals tend to show a greater decrease compared to athletes).[38]

ACE-031, a recombined protein that was made from the extracellular part of the ARIIB receptor combined with the Fc region of human immunoglobin

IgG1, which acts as a strong myostatin inhibitor, was used during a double blind placebo control study in 48 healthy, post-menopausal women. The drug was well tolerated (with a rash as the only adverse effect), and it achieved a statistically significant increase in muscle mass, while at the same time it improved bone and fat tissue metabolism.[40]

The anabolic hormone testosterone increased muscle mass, power, force and function; however, it caused water retention, an increase in hematocrit, short-term worsening of sleep apnea and an increase in the frequency of cardiovascular accidents, while it also appear to be dangerous in cases of prostatic hypertrophy. Selective androgen receptor modulators (SARMs) have succeeded in augmenting muscle mass with in addition a slight increase in muscle power, but it may worsen existing heart failure (Table). In other studies, the use of growth hormone was related to a positive nitrogen (N) balance and to increase of muscle mass; on the other hand, the patients developed joint pain, muscle pain, edema, carpal tunnel syndrome and hyperglycemia. Ghrelin (a hormone that increases appetite) agonists have been shown to augment muscle mass, however with adverse effects such as fatigue, dyspnea and atrial fibrillation. Antimyostatin antibodies appeared to increase fat-free muscle mass (lean body mass) and grip strength, though the following adverse effects were concurrently reported: allergic urticaria, aseptic meningitis, diarrhea, confusion and fatigue. After using activin type IIR receptor antagonists, there was an increase of muscle volume, as measured in the thigh, of muscle mass and of the distance a person could walk in 6 minutes, while the adverse effects included acne and involuntary muscle contractions (cramps). Perindropril, an angiotensin-converting-enzyme inhibitor, increased the distance that the patient could walk and decreased hip fracture frequency, although it caused hypotension, hyperkalemia, muscle cramps and numbness. The two latest drugs that have been tested are Pindolol, a non-selective beta blocker (antagonist) was able to preserve muscle mass and increased grip strength in the elderly, and Tirasemtiv, which seemed to improve muscle function. No significant adverse effects have been reported.[41]

Table. Selective androgen receptor modulators (SARMs) pre-clinical and clinical development

Drug	Commercial name	Status of study	Indication
MK2866 GTx-024	Ostarine Enobosarm	Phase II b Clinical study	Increase of muscle mass
LGD-4033	Ligandrol	Pre-clinical study	Improves muscle mass
971086	GSK 971086	Clinical study phase I (2011)	Androgen agonist
Karo		Stopped	

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