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Ciclo XXX

Multifold aspects of obesity and insulin resistance: comorbidities and crosstalk with thyroid gland

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Summary

Obesity is a complex condition associated with an increased risk of multiple comorbidities and endocrine abnormalities, including thyroid dysfunction. Recently, there has been increasing interest toward the association between thyroid function and obesity. Therefore, this thesis aims to investigate the complex metabolic phenotypes of obesity on one side and thyroid disorders on the other, then converging to the study of the crosstalk between these conditions.

Severe obesity is characterized by highly variable degrees of metabolic dysfunction. Insulin resistance is a common factor that, per se, predisposes to metabolic abnormalities and a chronic proinflammatory state. Beyond the well-known detrimental effects of chronic inflammation on cardio-metabolic health, our interest primarily focused on the characterization of multiple molecules and we documented a new role for ANGPTL8, vitamin D, uric acid and oxytocin in association with the metabolic setting and comorbidities related to obesity.

There is consistent evidence that adipose tissue accumulation and fat partitioning potentially interfere with thyroid tumorigenesis and function. In studying exogenous and endogenous factors involved in differentiated thyroid cancers (DTC) phenotypes, our analysis on two geographically distinct populations with DTCs show that many environmental factors could influence thyroid cancer histopathological features and clinical behaviour. Subsequently, in assessing the role of metabolic determinants on DTC features in the general population, we also obtained preliminary evidence of a potential key role for insulin resistance and pro-inflammatory adipokines on clinical profile of DTCs.

We then addressed the association between obesity and thyroid function in multiple ways, and showed that the metabolic milieu of obesity, as well as body weight changes, can significantly impact thyroid hormone levels via interactions with body composition. Retrospective analyses also allowed us to evaluate the effect of obesity on thyroid replacement therapy in obese hypothyroid patients, confirming the complex interplay linking obesity and thyroid function, both in health and in disease.

All the above-mentioned results were published or are submitted for publication in international peer-reviewed scientific journals, hoping to contribute to expand current knowledge on the crosstalk between obesity and the thyroid gland.

Sommario

L’obesità è una condizione clinica complessa, associata ad un rischio aumentato di sviluppare numerose comorbidità e disfunzioni endocrine, tra cui quelle tiroidee. Recentemente si è sviluppato un crescente interesse circa l’associazione tra funzione tiroidea e obesità. Pertanto, questa tesi ha lo scopo di indagare, da un lato, la complessità dei diversi fenotipi metabolici associati all’obesità e, dall’altro, le patologie tiroidee, per poi studiare l’associazione fra le due condizioni.

L’obesità severa è caratterizzata da diversi gradi di alterazioni metaboliche. L’insulino-resistenza è un fattore comune che, di per sé, predispone a disordini metabolici e ad uno stato pro-infiammatorio cronico. Oltre che sui noti effetti dannosi dell’infiammazione cronica sulla salute cardio-metabolica, il nostro interesse si è focalizzato principalmente sulla caratterizzazione di diverse molecole attribuendo un nuovo ruolo ad ANGPTL8, vitamina D, acido urico e ossitocina nell’interazione con il profilo metabolico e le comorbilità associate all’obesità.

Esistono prove consistenti del fatto che l'accumulo di tessuto adiposo e la sua distribuzione siano potenzialmente in grado di interferire con la tumorigenesi e la funzione tiroidea. Nello studio di fattori esogeni ed endogeni coinvolti nei fenotipi del carcinoma differenziato della tiroide (CDT), le nostre analisi su due popolazioni di pazienti geograficamente distinte, affette da CDT, mostrano che diversi fattori ambientali possono influenzare le caratteristiche istopatologiche e il comportamento clinico dei tumori tiroidei.

Successivamente, nel valutare il ruolo dei determinanti metabolici sulle caratteristiche del CDT nella popolazione generale, abbiamo ottenuto risultati preliminari circa il potenziale ruolo chiave dell'insulino-resistenza e delle adipochine pro-infiammatorie nel determinare il profilo clinico dei CDT.

Abbiamo poi studiato l'associazione tra obesità e funzione tiroidea su diversi livelli, dimostrando che il setting metabolico dell'obesità, così come le variazioni del peso corporeo, possono avere un impatto significativo sui livelli degli ormoni tiroidei attraverso molteplici interazioni con la composizione corporea.

Alcune analisi retrospettive ci hanno inoltre consentito di valutare l'effetto dell'obesità sulla terapia sostitutiva con LT4 in pazienti obesi ipotiroidei, confermando l'esistenza di una complessa interazione tra obesità e funzione tiroidea, sia in condizioni normali che patologiche.

Tutti i risultati sopra menzionati sono stati pubblicati o sono in fase di pubblicazione su riviste scientifiche internazionali, con l'intento di contribuire ad ampliare le conoscenze attuali sull'associazione tra obesità e tiroide.

General Introduction

Obesity is one of the most important health risks of our time. The prevalence of obesity has increased worldwide since the mid 1970s. Obesity is associated with an increased risk of diabetes, dyslipidemia, cardiovascular disease, sleep apnea, cancer and all-cause mortality *(Golden 2009)*. Moreover, severe obesity is an important cause of premature mortality among middle-aged adults *(Mehta 2009)*. Importantly, obesity, especially central obesity, is linked to many endocrine abnormalities *(Kokkoris 2003)*, including thyroid dysfunction *(Reinehr 2010)*.

Recently, there has been increasing interest toward the association between thyroid function and obesity. Based on the notion that triiodothyronine (T3) controls metabolic and energy homeostasis influencing body weight, thermogenesis, lipolysis and metabolism of cholesterol, and that thyroid-stimulating hormone (TSH) induces differentiation of preadipocytes into adipocytes and adipogenesis, thyroid function has been extensively investigated in obese adults *(Laurberg 2012, Biondi 2010)*. An elevated level of TSH with normal peripheral thyroid hormone concentration, potentially suggesting the occurrence of subclinical hypothyroidism, has been consistently found in obese subjects. Several mechanisms leading to hyperthyrotropinemia in obesity have been hypothesized, including subclinical hypothyroidism caused by iodine deficiency, autoimmune thyroiditis, mutations in TSH-R gene or stimulatory effects of leptin, the main adipocytokine, on TRH secretion *(Santini 2014)*. Moreover, several metabolic outcomes have been related to TSH elevation. In fact, TSH increase was shown to be associated with increased cholesterol and triglycerides and with decreased HDL-C. Moreover, thyroid hormones are important determinants of glucose homeostasis. In fact, increased thyroid hormone levels impair the ability of insulin to suppress hepatic glucose production and increase glucose uptake in muscles *(Mullur 2014)*. The presence of insulin resistance, which is a major component of obesity, was found to be associated with an increased thyroid volume and is an independent risk factor for thyroid nodule formation *(Rezzonico 2008, Ayturk 2009, Yasar 2011)*. Furthermore, the prevalence of insulin resistance is reckoned as a potential risk factor for thyroid tumorigenesis *(Rezzonico 2009, Marcello 2014)*, as well as for some non-thyroid cancers *(Djiogue 2013)*.

The increased TSH levels and concomitant changes in thyroid hormone levels, with FT3 levels that are usually in the upper normal range and FT4 levels in the lower normal range of obese subjects, may reflect an adaptation process aiming to increase energy expenditure and control further weight gain *(Reinehr 2010)*. Therefore, the changes of thyroid hormones concentration may be regarded as a consequence rather than a cause of obesity.

Aim of this thesis

Reflecting the doctoral research activity in the fields of obesity, thyroid diseases and their two-way interaction, this thesis aims to describe the results of studies on:

- Phenotypes, complications and treatments of obesity and metabolic disorders.
- Thyroid dysfunction and cancer in relation to metabolic setting and risk factors.
- The crosstalk between obesity and thyroid function.

CHAPTER 1

Obesity and metabolic disorders: Phenotypes, complications and treatments

SEVERE OBESITY:

METABOLIC FEATURES, PHENOTYPES AND COMPLICATIONS

Introduction

The prevalence of obesity has increased dramatically in the last decades and is now considered a major health problem *(Flegal 2010).* In fact, the current epidemic of obesity has been reported as the leading cause for the decreased life expectancy forecast for the next generation *(Lavie 2009)*. Worldwide, the prevalence rate for being overweight or obese between 1980 and 2013 increased 27.5% for adults and 47.1% for children, for a total of 2.1 billion individuals considered overweight or obese *(Ng 2014).* Body mass index (BMI), which is weight in kilograms divided by height in meters squared, is used to identify obesity. For adults, a BMI of 25.0 to 29.9 kg/m^2 is defined as overweight and a BMI of 30 kg/m^2 or higher is defined as obese *(Jensen 2014)*. For every 5-unit increase in BMI above 25 kg/m^2, overall mortality increases by 29%, vascular mortality by 41%, and diabetes-related mortality by 210% *(Whitlock 2009)*.

Patients with obesity are at increased risk of morbidity from hepatic steatosis, hypovitaminosis D, dyslipidemia, type 2 diabetes, hypertension, cardiovascular diseases, respiratory problems, sleep apnea, osteoarthritis, and some cancers *(Jensen 2014)*.

Aiming to explore the metabolic features, phenotypes and complication of severe obesity, the doctoral activity was focused on the following subjects:

- ANGPTL8 and liver steatosis in obesity and the Prader Willi syndrome (PWS)

- A role for adiponectin oligomers in modulating Vitamin D effects on glucose homoeostasis and insulin sensitivity in obesity
- Uric acid as a potential new marker of positive energy states in obesity
- Circulating oxytocin across menopause in the lean and obese state
- A rare case of endocrine-dependent obesity and related therapeutic strategies.

Circulating angiopoietin-like 8 (ANGPTL8) is a marker of liver steatosis and is negatively regulated by Prader-Willi Syndrome

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Abstract

Background. ANGPTL8 is a liver-derived protein related to insulin-sensitivity. Its relationship with obesity and liver function in Prader-Willi syndrome (PWS) is unknown. The present study investigated circulating ANGPTL8 in PWS and controls with common obesity, assessing its association to liver steatosis.

Methods. For this purpose, 20 obese PWS and 20 controls matched for body mass index (BMI), sex and age underwent analysis of ANGPTL8 levels, glucose and lipid metabolism. Liver function tests and degree of liver steatosis by ultrasonography (US), fat-free mass (FFM) and fat mass (FM) by dual-energy x-ray absorptiometry (DEXA) were also assessed.

Results. In comparison to controls, obese PWS showed lower values of FFM ($p<0.0001$) and higher FM ($p=0.01$), while harbouring higher HDL cholesterol, lower triglycerides and OGTT-derived insulin levels, as well as a lower prevalence and severity of liver steatosis. With respect to obese controls, ANGPTL8 levels were significantly lower in PWS ($p=0.007$) and overall correlated with transaminase levels and the severity of liver steatosis, as well as FFM ($p<0.05$ for all). By a stepwise multivariable regression analysis, ANGPTL8 levels were independently predicted by PWS status ($p= 0.01$) and liver steatosis ($p<0.05$).

Conclusions. ANGPTL8 levels are lower in PWS than obese controls and are inversely associated with the severity of liver steatosis. Further studies should investigate the potential genetic basis for this observation.

Background

Prader-Willi syndrome (PWS) is an imprinted neurobehavioral condition caused by the lack of expression of genes located on the paternal chromosome 15q11.2-q13. There are three main genetic subtypes in PWS: paternal 15q11-q13 deletion (65–75% of cases), maternal uniparental disomy of chromosome 15 (UPD15) (20–30% of cases), and imprinting defects (1–3%) *(Angulo 2015)*. The smallest deletions discovered to date demonstrate that the SNORD116 snoRNA gene cluster can explain much of the PWS phenotype *(Hasan 2016)*. Clinically, PWS is characterized by neonatal hypotonia and failure to thrive, cognitive and behavioural disorders, endocrine defects such as short stature and hypogonadism, autonomic dysregulation. PWS is tipically associated with a lack of satiety, which generates obsessive craving for food and results in extreme obesity by the adult age *(Cassidy 2009)*.

Adult patients with PWS show peculiar body and metabolic features. Compared to BMI-matched controls, PWS patients harbour a predominant accumulation of subcutaneous adipose tissue, with lower accumulation of visceral adipose tissue than that observed in patients with common obesity *(Goldstone 2001, Goldstone 2002, Tanaka 2013)*. In addition, lean body mass and muscle function is impaired *(Lafortuna 2014)*, and results in reduced resting energy expenditure (REE) and decreased voluntary activity *(Butler 2007)*. Despite this unfavourable body composition, the metabolic phenotype of PWS is characterized by lower insulin levels and higher insulin sensitivity as opposed to obese controls *(Talebizadeh 2005, Schuster 1996)*, while dyslipidaemia rarely occurs in PWS *(Sanchez-Ortiga 2012)*. Although the molecular mechanisms driving this peculiar metabolic profile are not well understood, it can be hypothesized that the elevation of orexigenic hormones, such as ghrelin (*Goldstone 2004, Prodam 2009)*, and different expression of adipocytokines *(Pagano 2005)*, particularly adiponectin *(Kennedy 2006)*, could intervene to regulate the metabolic profile of PWS adults *(Lacroix 2015)*.

A recently identified liver protein, angiopoietin-like 8 (ANGPTL8), has been described to be involved in different metabolic pathways related to glucose and lipid metabolism *(Tseng 2014a, Tseng 2014b, Rong Guo 2016)*. As summarized by Tseng et al., several researchers have investigated the subcellular localization of ANGPTL8, demonstrating that its cytoplasmic vesicle-like distribution is likely involved in the lipid regulation pathway

(Tseng 2014a). Serum ANGPTL8 has been detected in sera from humans and mice, with its levels found to be positively correlated with triglycerides (TG) and very low-density lipoprotein (VLDL) levels *(Tseng 2014b)*. Intracellular ANGPTL8 is associated with lipid droplets, suggesting that ANGPTL8 may serve as a lipoprotein and could be secreted or taken up with a lipid-associated compartment *(Tseng 2014b)*.

Regarding its function/s in glucose homeostasis, ANGPTL8 might be key in regulating postprandial glucose metabolism. In fact, liver ANGPTL8 over-expression in mice increases insulin-mediated synthesis of glycogen *(Rong Guo 2016)*. Moreover, it is able to promote the suppression of key enzymes involved in gluconeogenic pathways, thereby improving insulin resistance *(Rong Guo 2016)*.

To date, the metabolic significance of ANGPTL8 in human obesity and obese patients with PWS is unknown. As such, this study was designed to investigate the relationship between ANGPTL8 levels and adiposity, metabolic homeostasis and liver steatosis in association with obesity and the PWS condition.

Methods

Patients

This study enrolled 40 patients, consisting of 20 PWS adults with obesity (10 F/10 M; age, 34.2±7.6 years; BMI, 45.5±9.4 kg/m^2) and 20 BMI-matched control subjects (10 F/10 M; age, 35.0±8.3 years; BMI, 48.6±10.2 kg/m^2), referred to our institution for work-up and rehabilitation of obesity and its comorbidities. All PWS individuals received a diagnosis based on typical syndromic features confirmed by molecular genetic studies of chromosome 15, including 15q11-q13 deletion in 16 (10 males and 6 females) and UPD15 in the remaining 4 females. Exclusion criteria included any liver disease except for newly diagnosed steatosis, kidney failure, autoimmune diseases, uncontrolled hypothyroidism and/or diabetes mellitus, exposure to glucocorticoids or alcohol consumption. With respect to hormone replacement therapy in PWS, 9 patients were treated with rhGH, 2 female patients with estrogens and 2 patients with levothyroxine. Four PWS patients were treated with psychotropic medications. The experimental procedure was approved by the ad hoc Ethical Research Committee of the Istituto Auxologico Italiano, Verbania,

Italy. A written informed consent was obtained from the PWS patients and their parents or guardians, and from the obese participants. The study protocol conformed to the guidelines of the European Convention on Human Rights and Biomedicine concerning biomedical research.

Body measurements and instrumental tests

All subjects underwent body measurements wearing light underwear, in fasting conditions after voiding. Weight and height were measured to the nearest 0.1 kg and 0.1 cm, respectively, using standard methods. BMI was expressed as body mass (kg)/height $(m)^2$. Obesity was defined for any BMI over 30 kg/m^2 *(WHO 2000)*. Waist circumference (WC) was measured midway between the lowest rib and the top of the iliac crest after gentle expiration; hip measurements were taken at the greatest circumference around the nates.

A dual-energy x-ray absorptiometry (DEXA; GE Lunar, Madison, WI, USA) was performed for the assessment of body mass. This was expressed as lean body mass in kilograms and fat body mass as the percentage of total body mass.

The REE was expressed in kilocalories/24 h and determined in a thermoregulated room (22–24°C) by computed open-circuit indirect calorimetry, measuring resting oxygen uptake and resting carbon dioxide production by a ventilated canopy (Sensormedics, Milan, Italy) at 1-min intervals for 30 min, expressed as 24 h value.

In order to assess the presence and severity of liver steatosis, liver US was performed by the same operator who was blinded to the laboratory and clinical data at the time of the procedure, using a high-resolution US system (LOGIQ 7, GE Healthcare, Waukesha, WI, USA). The degree of hepatic steatosis was assessed semi-quantitatively on the basis of hepatorenal echo contrast, liver brightness, deep attenuation and vascular blurring. Liver steatosis was established by a validated method of US grading (categorized as: G0=absent; G1=mild; G2=moderate, G3=severe steatosis) *(Shannon 2011)*, to accomplish for the subjective difficulties of PWS patients to undergo invasive or radiological assessment (MRI) where patients' collaboration was needed.

Laboratory tests

Blood samples were drawn under fasting conditions, centrifuged, and stored at −80 °C until required.

Serum ANGPTL8 levels were assessed using a commercially available human EIA kit (Phoenix Pharmaceutics, Inc, Burlingame, CA, USA). The assay procedure was performed in accordance with the manufacturer's instructions. All samples were analyzed in duplicate. Intra-assay CV and inter-assay CV of ANGPTL8 were less than 10% and 15% respectively. Minimum detectable concentration was 0.12 ng/mL. Furthermore, the EIA was specific for human ANGPTL8. In addition, quality controls were included in all EIA measurements with the results within the expected range.

Routine laboratory data included levels of C-reactive protein, alkaline phosphatase, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT), glucose, total cholesterol, high-density (HDL) and low-density lipoprotein (LDL) cholesterol, triglycerides (TG) and glycated haemoglobin (HbA1c), measured by enzymatic methods (Roche Diagnostics, Mannheim, Germany). Levels of insulin were measured using a Cobas Integra 800 Autoanalyzer (Roche Diagnostics, Indianapolis, IN, USA). Ultrasensitive C-reactive protein (CRP) was measured by CRP (latex) HS Roche kit.

Glucose homeostasis was evaluated by oral glucose tolerance test (OGTT) in all subjects and glucose tolerance was expressed, according to ADA recommendations *(ADA 2012)*, as normal fasting plasma glucose (FPG) if <100 mg/ dl (5.6 mmol/l); impaired FPG (IFG) if FPG was 100–125 mg/dl (6.9 mmol/l); impaired glucose tolerance (IGT) if 2-h post-OGTT plasma glucose was 140–199 mg/dl (7.8–11.0 mmol/l); T2DM if FPG was ≥126 mg/dl (≥7 mmol/l) two days apart, or if 2-h post-OGTT plasma glucose was ≥200 mg/dl (≥11.1 mmol/l). HbA1c values of 5.7 and 6.5% were considered as the threshold of normal glucose metabolism and T2DM, respectively.

Matsuda *(Matsuda 1999, De Fronzo 2010)* and Stumvoll *(Stumvoll 2000)* indexes were used to measure insulin sensitivity obtained from plasma glucose and insulin concentrations during the oral glucose tolerance test (OGTT). The Matsuda index provides a reasonable index of whole-body insulin sensitivity *(Matsuda 1999, De Fronzo 2010)* and was calculated using the following formula: 10,000/square root of [(fasting glucose ×

fasting insulin) × (glucose × insulin at time 120 min during OGTT)]. The Stumvoll index is a predictor of individual's insulin sensitivity and β-cell function *(Stumvoll 2000)* and was calculated using the following formula: 0.156 − (0.0000459 × insulin at time 120 min during OGTT) − (0.000321 × fasting insulin) − (0.00541 × glucose at time 120 min during OGTT).

Insulin resistance was calculated by the homeostatic model of insulin resistance (HOMA-IR) index: insulin (mIU/L) × [glucose (mmol/L)/22.5] *(Matthews 1985)*. A HOMA-IR value greater than 2.0 was considered indicative of insulin resistance, as obtained in a sample of the Italian population *(Bonora 2000)*. The homeostatic model of β cell function (HOMA-B) was used to describe the functionality of pancreatic beta cells and calculated using the following formula: 20 × [insulin (mIU/L)/glucose (mmol/L) − 3.5] *(Matthews 1985)*.

Data analysis

Statistical analysis was performed using SPSS version 18 (Somers, NY, USA). Values are expressed as means ± standard deviation (SD). For comparative analysis, ANOVA between the 2 groups and paired-T test intra-groups were used. For comparative analysis of ANGPTL8 levels across liver steatosis categories, Kruskall-Wallis test with Dunn's correction was used. Pearson's correlation analysis and the Chi square were used to identify significant associations between variables of interest. Stepwise multivariate regression analysis was used to evaluate the independent association of variations in ANGPTL8 with metabolic, anthropometric or biochemical parameters. The multilinear model included PWS status (no PWS=0; PWS=1), liver steatosis or ALT levels, age, gender and BMI as independent variables. β coefficients and related significance values obtained from the models are reported. $P< 0.05$ was considered as statistically significant.

Results

A summary of anthropometric and biochemical data is reported in Tables 1 and 2 (pag. 23, 24), and Figure 1 (pag. 26). BMI values were comparable between the two groups and ranged, collectively, between 31.5–73.3 kg/m^2. Severe obesity (BMI > 40 kg/m^2) was present in 67.5% of cases (13 PWS and 14 obese patients). Abnormal glucose metabolism

was detected in 50% of PWS and 55% of obese controls (T2DM in 25% of PWS and 20% of obese subjects, IGT in 20% of PWS and 35% of obese controls, IFG in 5% of PWS and no controls).

Anthropometric parameters differed between groups. PWS showed lower values of REE ($p<0.0001$) and FFM ($p<0.0001$), and higher %FM ($p=0.01$) with respect to obese controls. Nevertheless, in the PWS group we observed lower OGTT-derived insulin ($p=0.02$), higher HDL ($p=0.02$ for all) and lower TG ($p=0.01$) and urate levels ($p=0.004$), which was suggestive of a better metabolic profile in PWS compared to obese controls.

Liver function tests and liver steatosis evaluated by US resulted less impaired in PWS than obese controls. In fact, transaminases levels were high (>40 U/L) in 3 PWS and in 7 obese patients (15% vs. 35%), while US documented liver steatosis in 14 PWS and 18 obese controls (70% vs. 90%; $\chi^2=12.5$, $p<0.001$). According to the grading of liver steatosis, PWS had a higher prevalence of G0 (30% vs. 7%; $\chi^2=17.5$, $p<0.0001$), a similar prevalence of G1 (20% vs. 20%, respectively) and G2 (30% vs. 33%, respectively), and a lower prevalence of G3 score (20% vs. 40%; $\chi^2=9.5$, $p<0.01$), when compared with obese controls.

Analysis of circulating ANGPTL8 showed overall significantly lower levels in PWS compared to obese controls (Figure 2, pag. 27). ANGPTL8 levels were only modestly overlapping between populations and were less variable in PWS than obese subjects. The greater dispersion around the mean of ANGPTL8 values in the obese group likely reflected differences in liver function, as confirmed by an elevation of AST and ALT levels in both outliers of the obese group of controls (Figure 2, pag. 27). Corroborating these findings, correlation analysis in the two groups as a whole (Table 3, pag. 25) showed significant positive correlations between ANGPTL8 and AST ($r=0.35$, $p<0.05$), ALT ($r=0.38$, $p=0.01$), as well as with the grading of liver steatosis ($r=0.36$, $p<0.05$). These relationships were further substantiated by the observation of parallel increases of ANGPTL8 levels with the severity of liver steatosis ($p=0.01$ by repeated measures ANOVA) (Figure 3, pag. 28), and by association observed between ANGPTL8 levels and liver steatosis in the group of obese controls ($r=0.53$, $p<0.05$). Of note, a significant association was also seen between ANGPTL8 levels and REE ($r=0.32$, $p=0.05$) as well as FFM ($r=0.31$, $p<0.05$), while only in PWS ANGPTL8 levels were inversely associated with %FM ($r=-0.46$, $p<0.05$). Significant correlations obtained on the entire dataset were lost after controlling for PWS status, thereby confirming the blunting effect of PWS on ANGPTL8 levels ($r=-0.42$, $p<0.01$). There

were no differences in ANGPTL8 levels and liver steatosis when PWS group was analysed according to GH treatment or genotype (data not shown).

Stepwise multivariable regression analysis documented that ANGPTL8 levels were negatively predicted by PWS status (standardized $\beta=-0.41$, $p=0.01$). After the removal of PWS status from the regression equation, ALT levels (standardized $\beta=0.39$, $p=0.01$) or the score of liver steatosis (standardized $\beta=0.35$, $p<0.05$), acted as independent predictors of ANGPTL8 levels.

Discussion

The present study analysed the association between ANGPTL8 levels and adiposity, metabolic profile and liver steatosis in relation to the adult PWS condition and obesity. Our results show that PWS patients harbor lower ANGPTL8 levels than obese controls and that ANGPTL8 levels are more closely associated with liver steatosis, than with body composition and metabolic homeostasis.

The metabolic phenotype of PWS is different as compared to common obesity and some metabolic complications typically related to obesity, such as insulin resistance and reduced hepatic insulin extraction, are less severe than expected for the degree of fat accumulation *(Grugni 2016)*. In addition, PWS is associated with peripheral rather than central distribution of body fat *(Goldstone 2001)*, less severe metabolic signatures in adipocytes *(Lacroix 2015)*, and abnormalities in growth hormone secretion, ghrelin levels and adipokine patterns, when compared to common obesity *(Goldstone 2004, Pagano 2005, Kennedy 2006, Lacroix 2015)*. From a clinical viewpoint, factors influencing these discrepancies are incompletely understood.

ANGPTL8 is a liver and adipose tissue-produced protein, involved in the regulation of triglyceride and glucose metabolism. Its activity involves the ability of reducing serum triglyceride clearance and improving insulin resistance *(Tseng 2014a, Quagliarini 2012, Ren 2012, Zhang 2012, Fu 2013, Zhang 2013)*. In the current study, we observed lower circulating levels and less interindividual variability of ANGPTL8 in PWS when compared to subjects with common obesity. While insulin resistance and whole-body insulin sensitivity index did not greatly differ between PWS and obese controls, several anthropometric and metabolic differences existed between the two populations.

Particularly, PWS patients showed higher HDL cholesterol and lower TG levels than their control counterpart. Previous studies have suggested that PWS is characterized by a more efficient triglyceride storage likely due to an increase of adipose tissue lipoprotein lipase (LPL) activity, suggesting an altered pathway of fat mobilization and oxidation *(Meaney 1989)*. Noteworthy, ANGPTL8 shows the ability to suppress triglyceride clearance through the inhibition of LPL, thus increasing serum triglycerides *(Zhang 2013)*. Therefore, we speculate that the decreased serum ANGPTL8 levels in PWS could contribute in explaining the lower triglyceride levels seen in PWS when compared to obese controls. In our study groups, the role of circulating ANGPTL8 on glucose homeostasis and insulin resistance appeared to be of little relevance compared to data in the literature *(Rong Guo 2016)*. In fact, no correlations between ANGPTL8 and glucometabolic parameters were observed. These results do not disagree with recent data, but rather suggest that ANGPTL8 is not as robustly involved in β-cell proliferation as originally proposed *(Cox 2016)*, at least in our study populations.

In the search for mechanisms to explain our observations, we noted that circulating ANGPTL8 paralleled the behaviour of crucial determinants of metabolic health, such as liver steatosis. In fact, circulating ANGPTL8 levels were negatively associated with indices of liver steatosis, i.e. transaminases and US-derived scores of steatosis, which are recognized non-invasive markers of liver impairment in obesity *(Clark 2003, Bedogni 2014, Marchesini 2003)*. While liver biopsy is the gold-standard method for the accurate staging of non-alcoholic liver steatosis, several previous studies demonstrated a strong correlation between US findings and the degree of liver steatosis documented by biopsy *(Shannon 2011, Dasarathy 2009, Saadeh 2002)*. Bearing in mind the limitation of our approach, current data confirm that liver steatosis is less severe in PWS than in common obesity *(Bedogni 2014)*, and substantiates the emerging role of liver steatosis on circulating ANGPTL8 in severe obesity, which is further strengthened by the results of correlation analyses and multivariable regression analyses. Being a primarily liver-produced protein, ANGPTL8 is positively associated with biochemical indices of liver injury and steatosis in overweight and obese individuals *(Guo 2015)*. In hepatoma cells, Tseng YH et al. demonstrated that ANGPTL8 is mainly localized in the cytoplasm with a vesicle-like distribution *(Tseng 2014b)*, possibly implying that hepatocyte lysis linked to steatosis could promote the leakage of ANGPTL8 vesicle in the bloodstream, thus helping to

explain our findings. Complementing literature data *(Guo 2015, Lee 2016)*, our results suggest that ANGPTL8 levels could act as a novel surrogate biomarker for liver steatosis in non-PWS obese individuals. As certain adipokines can predict the severity of liver steatosis *(Jamali 2016)*, however, studies are required to clarify the association between adipocytokines and ANGPTL8 levels in steatosis.

Conclusions

In conclusion, ANGPTL8 levels are lower in PWS than obese controls and, overall, they seem to reflect the severity of liver steatosis. Further studies should investigate the potential genetic basis of these findings.

Tables

Table 1. Summary of anthropometric data obtained in PWS subjects and obese controls. Data are expressed as mean ± SD. Comparison between populations was performed by ANOVA test. Significant differences are shown in bold characters.

Variables	PWS (n= 20)	Obese (n= 20)	P Value
Males/females	10/10	10/10	—
Age (years)	34.2 ± 7.6	35.0 ± 8.3	0.7
BMI (kg/m^2)	45.5 ± 9.4	48.6 ± 10.2	0.3
Weight (Kg)	**105.9 ± 23.5**	**137.6 ± 27.2**	**<0.001**
Height (cm)	**152.6 ± 8.1**	**168.5 ± 10.5**	**<0.0001**
Waist (cm)	124.8 ± 15.2	132.6 ± 12.8	0.09
Hip (cm)	130.1 ± 16.8	140.4 ± 18.8	0.08
Waist-to-hip ratio	0.96 ± 0.08	0.95 ± 0.11	0.7
FM (%)	**53.7 ± 6.1**	**47.5 ± 8.1**	**0.01**
FFM (Kg)	**47.8 ± 11.6**	**70.2 ± 13.7**	**<0.0001**
REE (kcal/day)	**1607.5 ± 281.3**	**2198.6 ± 402.1**	**<0.0001**

BMI, body mass index; FM, fat mass; FFM, fat free mass; REE, resting energy expenditure.

Table 2. Summary of biochemical data obtained in PWS subjects and obese controls. Data are expressed as mean ± SD. Comparison between populations was performed by ANOVA test. Significance is shown in bold characters.

Variables	PWS (n= 20)	Obese (n= 20)	P Value
ANGPTL8 (ng/mL)	**0.58** ± **0.21**	**0.93** ± **0.50**	**0.007**
Glucose $OGTT_0$ (mg/dL)	100.6 ± 31.6	90.6 ± 10.0	0.1
Glucose $OGTT_{120}$ (mg/dL)	125.1 ± 44.9	142.2 ± 47.8	0.3
Insulin $OGTT_0$ (mIU/L)	10.7 ± 5.0	13.5 ± 6.2	0.1
Insulin $OGTT_{120}$ (mIU/L)	**53.4** ± **28.1**	**106.6** ± **86.5**	**0.02**
C- Peptide (µg/L)	**2.3** ± **0.9**	**3.2** ± **0.9**	**0.002**
Matsuda	6.39 ± 6.37	3.51 ± 2.25	0.06
Stumvoll	**0.077** ± **0.029**	**0.051** ± **0.045**	**0.05**
HOMA-IR	2.6 ± 1.6	3.0 ± 1.5	0.4
HOMA-B	149.4 ± 57.0	194.9 ± 109.3	0.1
HbA1c (%)	5.8 ± 1.0	5.7 ± 0.5	0.5
AST (U/L)	**18.9** ± **6.7**	**29.1** ± **15.1**	**0.01**
ALT (U/L)	**25.0** ± **16.9**	**40.5** ± **29.9**	**0.02**
GGT (U/L)	36.9 ± 50.0	34.0 ± 26.6	0.8
ALP (U/L)	77.1 ± 19.6	73.7 ± 14.9	0.5
CHO (mg/dL)	178.6 ± 42.6	186.9 ± 25.2	0.4
LDL CHO (mg/dL)	118.9 ± 39.3	120.4 ± 21.9	0.8
HDL CHO (mg/dL)	**48.9** ± **11.1**	**41.1** ± **9.2**	**0.02**
TG (mg/dL)	**97.3** ± **35.1**	**131.6** ± **45.1**	**0.01**
Urate (mg/dL)	**5.4** ± **1.0**	**6.5** ± **1.1**	**0.004**
CRP (mg/dL)	1.2 ± 1.3	1.1 ± 0.7	0.7

OGTT, Oral Glucose Tolerance Test; OGTT0 and OGTT120, OGTT at time 0 min and 120 min; HOMA-IR, homeostatic model of insulin resistance; HOMA-B, homeostatic model of β cell function; HbA1c, glycated haemoglobin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma glutamyl transpeptidase; ALP, alkaline phosphatase; CHO, total cholesterol; LDL CHO, low density lipoprotein cholesterol; HDL CHO, high density lipoprotein cholesterol; TG, triglycerides; CRP, C-reactive protein.

Table 3. Pearson's correlation analysis between ANGPTL8 levels and anthropometric and biochemical parameters in the study populations as a whole. For obese status: PWS = 1, common obese = 0. Significance is shown in bold characters.

	ANGPTL8 levels	
Parameters	**r**	**p-value**
Age (years)	0.04	0.7
Status	**−0.42**	**0.007**
BMI (kg/m^2)	−0.04	0.7
FM (%)	−0.26	0.1
FFM (kg)	**0.31**	**0.04**
REE (kcal/day)	**0.32**	**0.05**
Glucose $OGTT_0$ (mg/dL)	−0.10	0.5
Glucose $OGTT_{120}$ (mg/dL)	0.30	0.08
Insulin $OGTT_0$ (mIU/L)	0.09	0.5
Insulin $OGTT_{120}$ (mIU/L)	0.30	0.08
C- Peptide (μg/L)	0.30	0.07
AST (U/L)	**0.35**	**0.02**
ALT (U/L)	**0.38**	**0.01**
Liver steatosis score	**0.35**	**0.03**

BMI, body mass index; FM, fat mass; FFM, fat free mass; REE, resting energy expenditure; OGTT, Oral Glucose Tolerance Test; OGTT0 and OGTT120, OGTT at time 0 min and 120 min; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

Figures

Figure 1. OGTT-derived glucose levels (mg/dl) (A) and insulin levels (mIU/L) (B) obtained at time 0 min and 120 min in PWS and controls. Intra-groups differences were assessed by paired T-test. Inter-group analyses were performed by ANOVA. For significance: *p< 0.0001 by paired T-test; §p= 0.02 by ANOVA.

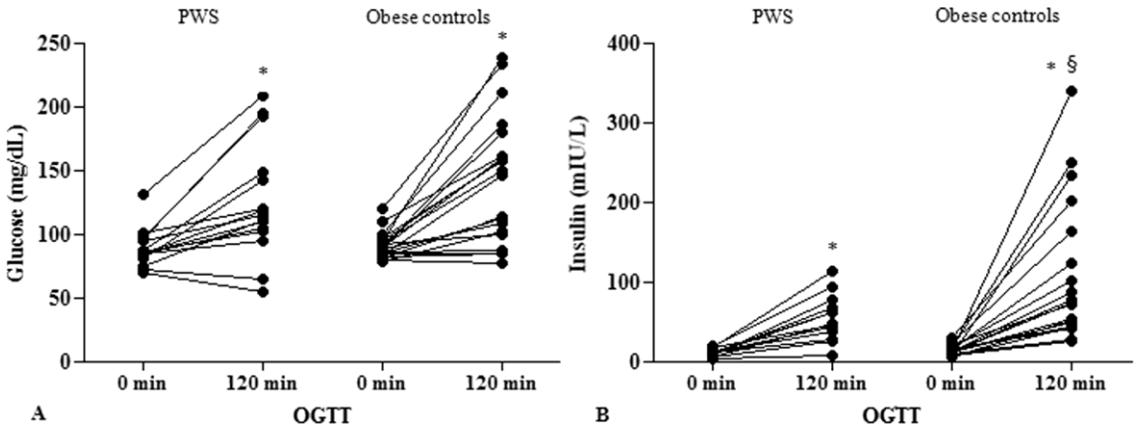


Figure 2. Individual values of circulating ANGPTL8 levels obtained in PWS patients and obese controls. Lines represent mean and standard deviation values in the two populations.

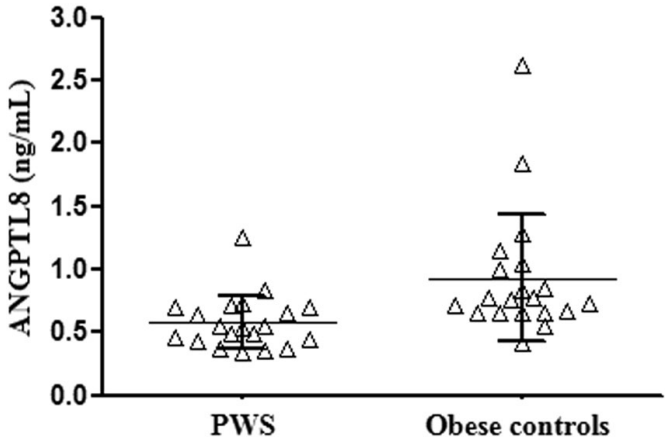
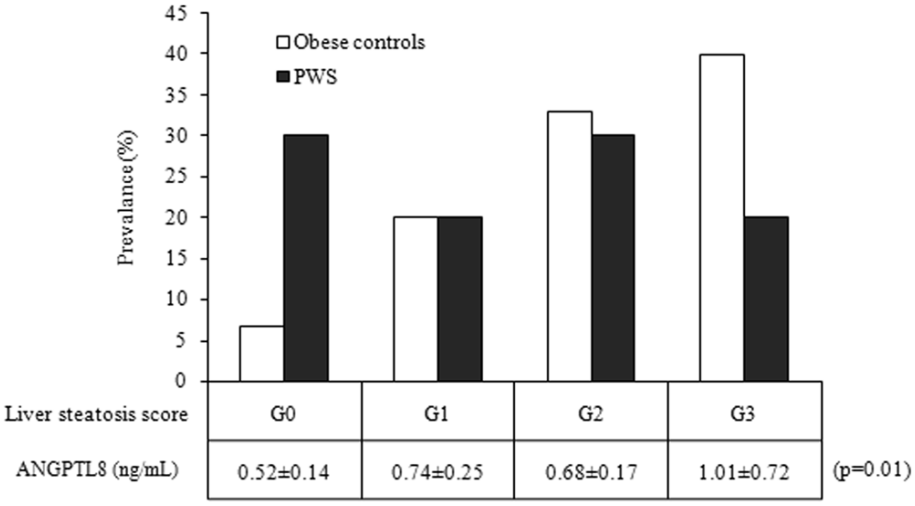


Figure 3. Histogram illustrating the prevalence (%) of liver steatosis in PWS and obese controls, and the corresponding ANGPTL8 levels across liver steatosis scores as obtained in the two population as a whole. Significance is expressed as obtained by ANOVA. Inter-group differences are listed in the Results section.



Acute Vitamin D3 Supplementation in Severe Obesity: Evaluation of Multimeric Adiponectin

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Abstract

Obesity predisposes to vitamin D deficiency (VDD) and glucose abnormalities. It is currently debated if vitamin D administration may improve glucose homeostasis by interacting with modulators of insulin sensitivity, such as adiponectin and its oligomers. In a 4-week inpatient study on a metabolic rehabilitation program, consisting of individualized caloric restriction and aerobic physical exercise in obese subjects with VDD, we assessed the acute effects of 600,000 IU cholecalciferol given per os VD group, 12 subjects; body mass index (BMI) 42.7±1.3 kg/m^2) or placebo per os (PL group, 12 subjects, BMI 39.8±0.9 kg/m^2) on high (HWM-A), medium (MMW-A), and low molecular weight adiponectin (LMW-A), as quantified by western immunoblot (WIB) and ELISA. During the 4-week study, dieting promoted a similar magnitude of weight loss in VD and PL groups. Compared to the PL group, cholecalciferol administration increased 25(OH)Vit D levels ($p<0.001$) and promoted a significant increase of HMW-A expression analyzed by WIB ($p=0.02$). In parallel, a significant decrease of leptin/HMW-A ratio ($p<0.05$), a biomarker of metabolic homeostasis, was observed. During the study, changes of MMW-A and LMW-A occurred independently of cholecalciferol administration, and were likely explained by weight loss. At odds with these findings, the ELISA assessment of adiponectin oligomers showed no modifications in the VD group or PL group. Current findings suggest that acute cholecalciferol administration selectively modifies HMW-A and the leptin/HMW-A ratio.

Background

Vitamin D is a fat-soluble secosteroid hormone that has an established physiological role in mineral and bone homeostasis. Its precursor vitamin D3, or cholecalciferol, is synthesized by 7-dehydrocholesterol in the skin during sun exposure, and it is hydroxylated first in the liver to form 25-hydroxycholecalciferol (calcidiol, or 25(OH)Vit D), and then in the kidney to generate the bioactive form 1,25(OH)2 Vit D, the actions of which are mediated by the vitamin D nuclear receptor (VDR). Notably, the VDR is widely expressed in human tissues, where it likely mediates the pleiotropic functions of vitamin D *(Holick 2007)*. It has long been shown that serum 25(OH)Vit D concentrations are inversely correlated with body mass index (BMI), as well as with several anthropometric or biochemical surrogates of adiposity *(Ding 2012)*. Vitamin D deficiency (VDD) affects up to 95% of patients with severe obesity *(Bellan 2014)*, and an associative network relates VDD and obesity to low grade inflammatory state, insulin resistance, metabolic syndrome, and type 2 diabetes mellitus (T2DM) *(Bellan 2014, McGIll 2008, Palomer 2008)*. Evidence collected in case-control and observational studies led to the hypothesis that cholecalciferol administration could improve glucose tolerance, pancreatic beta-cell function, insulin and C-peptide responses, as well as insulin sensitivity *(Mathieu 2005, Baziar 2014, Breslavsky 2013)*. However, the possibility that vitamin D3 supplementation may help to prevent the risk of T2DM has been challenged, and the ability of vitamin D3 supplementation to improve fasting glucose, HbA1c levels, or insulin resistance in patients with impaired glucose tolerance (IGT), gestational diabetes, or T2DM is openly debated *(Krul-Poel 2017)*.

Being a source of adipokines, white adipose tissue (WAT) is actively involved in regulating many endocrine and metabolic functions related to energy storage, glucose homeostasis, and cardio-metabolic health *(Athyros 2010)*. In particular, adiponectin is a WAT-derived adipokine that acts as an insulin-sensitizer and is capable of yielding anti-inflammatory and anti-atherogenic effects *(Matsuzawa 2005)*. Because the secretion of adiponectin is negatively regulated by adiposity, particularly visceral fat accumulation *(Han 2007)*, adiponectin levels are reduced in overweight/obese and insulin resistant states *(Shibata 2009)*, and increase upon bodyweight reduction *(Bobbert 2005)*. It has long been shown that adiponectin circulates as three isoforms with largely different molecular weights. The

high molecular weight adiponectin isoform (HMW-A) is the recognized major determinant of insulin-sensitizing effects of adiponectin in liver, muscle, and endothelial tissue, as compared to medium (MMW-A) and low molecular weight adiponectin (LMW-A) *(Kaser 2008, Antoniades 2009).* In previous investigations, serum HMW-A expression was positively associated with indices of insulin sensitivity and high density lipoprotein (HDL) cholesterol, while being negatively associated with BMI, central body fat, hyperinsulinemia, and T2DM *(Lara-Castro 2006, Tschritter 2003, Basu 2007)*. In obesity, HMW-A levels are significantly decreased but increase during weight loss *(Mai 2014, Polak 2007)*. Cross-sectional studies found a positive association between circulating adiponectin and 25(OH)Vit D levels *(Liu 2009, Vaidya 2012, Al-Daghri 2013)*, as well as between VDD and hypoadiponectinemia *(Gannagè-Yared 2009, Nimitphong 2009)*. While meta-analytic data failed to prove stimulatory effects of cholecalciferol supplementation on total adiponectin concentrations *(Dinca 2016)*, it has been shown in obese children that VDD is associated with lower expression of adiponectin isoforms, particularly HMW-A, while 1 year supplementation with cholecalciferol was shown to upregulate HMW-A expression independent of changes in BMI *(Walker 2014)*. Pediatric studies also found that the increase in HMW-A levels was correlated with improvements in insulin sensitivity *(Gueugnon 2012, Martos-Moren 2010)*. Finally, cholecalciferol supplementation in children was shown to decrease the value of the leptin-to-adiponectin ratio (L/A) *(Belenchia 2013)*, a marker of metabolic disease *(Satoh 2004, Kotani 2005)*, which is better correlated with insulin resistance than the levels of each single peptide *(Inoue 2005, Inoue 2006)*.

Based on the evidence that cholecalciferol may improve insulin sensitivity, we sought to explore the effect of 25(OH)Vit D on glucose homoeostasis in non-diabetic obese subjects with VDD through analysis of indirect mediators of insulin sensitivity, such as adiponectin oligomers. We therefore undertook a placebo-controlled investigation to explore if the administration of a large dose of cholecalciferol in severely obese individuals could acutely modify the profile of adiponectin oligomers measured by western immunoblot and ELISA, in relation to anthropometric measures, indices of insulin resistance, and the leptin-to-(multimeric) adiponectin ratio.

Methods

Subjects

The study enrolled 24 obese patients (13 males/11 females; age 37.5±1.9 years; BMI 41.3±0.8 kg/m^2) who were referred to our institution for work-up and rehabilitation of obesity and its comorbidities. All patients were enrolled between July 2014 and February 2015 after biochemical demonstration of VDD status and were distributed during the three subsequent seasons. Written consent was obtained from all patients after full explanation of the purpose and nature of the study. The investigation was approved by the local ethical committee (Comitato Etico, Istituto Auxologico Italiano, Milano, Italy, 18F101_2011), and was developed in accordance with the 3rd edition of the Guidelines on the Practice of Ethical Committees in Medical Research.

Patients were otherwise healthy, and exclusion criteria included T1DM or T2DM, autoimmune or inflammatory diseases, kidney or cardiac disorders, chronic steroid treatment, any therapy capable of influencing calcium metabolism, vitamin D treatment, and comorbidities affecting 25(OH)Vit D metabolism, and menopause. Patients had not been prescribed any pharmacological therapies, diet therapy, dietary supplements, or anti-obesity compounds for at least 3 months prior to entering the study. Obese subjects were studied at baseline upon admission and following a four-week inpatient metabolic rehabilitation consisting of individualized caloric restriction equivalent to 75% of basal resting energy expenditure measured by indirect calorimetry, physical exercise comprising three sessions per week of aerobic activity, supported by a nutrition and lifestyle action program consisting of three 1-h classes per week dedicated to an educational approach on dietary behavior, nutrition knowledge, and motor activity. A standard inpatient hypocaloric diet consisting of 30% lipids, 50% carbohydrates, and 20% proteins was administered during the study, and the average caloric deficit was 488±79 kcal/day for the VD group and 485±83 kcal/day for the PL group. Neither supplements nor anti-obesity therapies were used during the study period.

Study Design

On admission, patients underwent routine analysis and assessment of VDD considered as 25(OH) Vit D levels <20 ng/mL *(Holick 2011)*. Subsequently, patients were randomly allocated to a single blind, placebo–controlled study. The starting study population included 26 subjects with VDD, two of whom dropped out during the study. The remaining 24 patients were divided between the vitamin D group (VD group; 6 males/6 females; age 38±2.4 years; BMI 42.7±1.3 kg/m^2) and the placebo group (PL group; 7 males/5 females; age 37±3 years; BMI 39.8±0.9 kg/m^2). An effort was made to include patients with comparable age, gender, and BMI in the two groups. Patients of the VD group underwent administration of an oral dose of commercially available oily solution containing 600,000 IU of cholecalciferol. Patients of the PL group were administered an equivalent volume of certified cholecalciferol-free olive oil. Following administration, fasting blood samples were collected for analysis at baseline and after 3, 7, 14, and 28 days post-administration in each group.

Body Measurements

All subjects underwent body measurement wearing light underwear in fasting conditions after voiding. Weight and height were measured to the nearest 0.1 kg and 0.1 cm respectively. BMI was expressed as body mass (kg)/height $(m)^2$. Obesity was defined for any BMI over 30 kg/m^2. Waist circumference was measured midway between the lowest rib and the top of the iliac crest after gentle expiration; hip circumference was measured as the greatest circumference around the nates. Anthropometric data were expressed as the mean of two measurements and were assessed at study entry and at study end. At baseline, all subjects underwent dual-energy X-ray absorptiometry (GE-Lunar, Madison, WI, USA) for measurement of lean and fat body mass the morning after an overnight fasting and after voiding.

Western Immunoblot (WIB) of Multimeric Adiponectin

Blood samples were drawn in fasting conditions, then they were centrifuged, separated, aliquoted, and kept at −80◦C until assays in single batches. As previously published *(Walker 2014)*, for WIB all serum samples were size-fractionated on 10% Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) under non-reducing (NR) conditions and electro-transferred to immuno-blot polyvinylidene difluoride (PVDF) membranes (BioRad, Hercules, CA, USA). Membranes were incubated with monoclonal anti-adiponectin (Adipogen, Incheon, Korea) and detected with the appropriate horseradish peroxidase-conjugated secondary antibody (Chemicon Millipore, Temecula, CA, USA).

Immunoreactive proteins were detected using enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL, USA) with image capture performed using a CCD-camera linked to ChemiTouch (BioRad). The results were quantified using Image Lab software (BioRad). Values are presented as arbitrary units (AU) normalized to total protein determined by Ponceau S staining (Sigma Aldrich, St. Louis, MO, USA).

Adiponectin Immunoassay

Serum total adiponectin and adiponectin multimeric forms were determined as previously published *(Mai 2014)*, using the Adiponectin (Multimeric) Enzyme Immunoassay (ALPCO Diagnostics, Salem, NH, USA), according to the manufacturer's instructions. Assay sensitivity is 0.019 ng/mL, the limit of detection performed is 0.0196 ng/mL; as reported by the manufacturer, overall intra- and inter-assay coefficient of variations (CV) for total, HMW, and MMW + HMW are 5.4–5.0%, 5.0–5.7%, and 5.0–6.0%, respectively. Total adiponectin and HMW-A were measured directly from the assay plates, whereas MMW-A and LMW-A were calculated; MMW-A was obtained by subtracting the concentration of HMW-A from the combined concentration of MMW-A and HMW-A (measured directly), and LMW-A was obtained by subtracting the combined concentration of MMW-A and HMW-A (measured directly) from the total concentration of adiponectin. In parallel to sample preparation, duplicate P1 (protease one of the kit)

digestions were also prepared for WIB under non-reduced conditions according to the manufacturer's instructions.

Biochemistry

Laboratory data were obtained in a central laboratory. Blood glucose and HbA1c were measured by enzymatic methods (Roche Molecular Biochemicals, Mannheim, Germany). HbA1c values of 6.5% were considered diagnostic for T2DM. Insulin (CVs, 0.8–1.5% and 2.4–4.9%) was measured using a Cobas Integra 800 Autoanalyzer (Roche Diagnostics, Indianapolis, IN, USA). In both groups, insulin resistance was calculated by the homeostatic model of insulin resistance (HOMA-IR) index as insulin (mU/L) × (glucose (mmol/L)/22.5). 25(OH)Vit D levels were determined by an Agilent HP1100 series HPLC system equipped with a Variable Wavelength Detector; the detector wavelength was set at 265 nm. 25(OH)-Vit D was determined using an analysis kit from Chromsystems (Chromsystems Instruments & Chemicals GmbH, Gräfelfing, Germany). Calibration was performed using a lyophilized serum calibrator NIST traceable of known concentration. Low and high concentration lyophilized sera samples were used as quality controls. Samples, calibrators, and quality controls were prepared according to the manufacturer's instructions. Assay sensitivity was 1.4 µg/L and the inter- and intra-assay coefficients of variations were 0.9–3.0% and 2.3–3.3%, respectively. Serum leptin levels were measured using a Leptin Human ELISA kit (Mediagnost, Ruetlingen, Germany) with a sensitivity of 0.2 ng/mL, and inter- and intra-assay coefficients of variability below 10%, as reported by the manufacturer.

Statistics

Data are expressed as the mean ± SEM. Statistical analyses were carried out by GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) using unpaired and paired two-tailed Student's t-test to compare baseline characteristics of the two groups and within the obese population at the different time-points. One-way ANOVA for repeated measures was used to assess within-group modifications of measured variables during the study. Two-way ANOVA was performed to analyze the effect of time, treatment, and time

treatment. Assessments of differences between groups were additionally assessed by the χ^2 test. Linear regression per Pearson's analysis was performed to determine correlation coefficients between different parameters. $p<0.05$ was considered as statistically significant. Percent Δ value was calculated as the final value − baseline value/baseline value × 100.

Results

Metabolic Effects of Cholecalciferol Supplementation

The characteristics of the 24 participants enrolled in the two study groups are shown in Table 1 (pag. 43). We found no difference in anthropometric variables between the VD group and the PL group at baseline, including BMI and body weight. A slight but non-significant difference in BMI ($p=0.08$) and percent fat mass ($p=0.07$) occurred between groups. Baseline levels of 25(OH)Vit D were similar across groups, and all subjects were 25(OH)Vit D deficient, considered as 25(OH)Vit D <20 ng/mL. Metabolic analysis showed baseline glucose levels in the diabetic range in one patient in the VD group. Baseline values of leptin and total adiponectin, as well as their ratio, were comparable between groups. Figure 1 (pag. 45) shows changes in 25(OH)Vit D levels following cholecalciferol or placebo administration in the study populations.

Figure 1 (pag. 45) shows the changes in serum 25(OH)Vit D levels induced by cholecalciferol or placebo supplementation in the studied populations. A significant change in 25(OH)Vit D levels occurred throughout the entire observation period only in the VD group, and analysis by repeated measures ANOVA showed that this change was significant ($p<0.001$). In particular, a sharp and significant increase in 25(OH)Vit D levels was observed as soon as after 3 days following administration and persisted at significantly higher levels than baseline throughout the study period. By contrast, there was no modification in 25(OH)Vit D levels in the PL group. The interaction between cholecalciferol treatment and time was significant ($p<0.0001$). At the end of the treatment period, obese patients treated with cholecalciferol lost 6±0.7% of their body weight (Table1, pag. 43), which did not statistically differ from the 5.5±0.5% weight loss

observed in the PL group. Overall changes of immunoassayable total adiponectin levels were negligible, whereas leptin levels significantly decreased in both groups. Hence, a significant reduction was also documented in the L/A ratio in both groups.

Analysis of Multimeric Adiponectin

Sera obtained from obese subjects undergoing the two treatment arms were processed by WIB to assess whether cholecalciferol treatment influenced adiponectin multimerization as compared to placebo. Despite a visual difference in baseline MMW-A values, analysis of multimeric adiponectin at study entry showed no difference between groups in the expression levels of HMW-A, MMW-A, and LMW-A. During the study, individual HMW-A profiles displayed a progressive increase in 71% of patients treated with cholecalciferol compared to 36% of those of the PL group (χ^2=24.6, p<0.0001). A cumulative increase in HMW-A expression, depicted in Figures 2 and 3 (pag. 46, 47), was only evident for the VD group but reached statistical significance 14 days following cholecalciferol administration (p<0.05 vs. baseline, 3 and 7 days). Inversely, we failed to document any significant interaction between time and treatment, probably due to the low sample number. An incremental trend was also observed for MMW-A and LMW-A, but this occurred in both groups, regardless of cholecalciferol treatment. Specifically, a significant change in MMW-A and LMW-A expression was achieved at 28-day when compared to the day 3 time-point (p<0.05), thus likely suggesting a diet-related effect.
Based on WIB analysis, we then sought to corroborate results obtained at baseline and after 14 and 28 days by multimeric adiponectin immunoassays. As shown in Table 2 (pag. 44), we failed to document significant increments in HMW-A in the VD group, while in the PL group there was a slight decrease of HMW-A at 28-day. By contrast, MMW-A and LMW-A did not appear to consistently change in either group during the experimental period. For all three adiponectin oligomers, we did not document significant effects of cholecalciferol administration, time of treatment, and their interaction by two-way ANOVA. Overall, one-way ANOVA for repeated measures also showed no significant difference. When the leptin/HMW-A ratio was used as a surrogate measure of the bioactive adiponectin multimerization, a decrease was only documented in the VD group (Table 1, pag. 43).

No correlation was observed when we analyzed the effect of vitamin D3 treatment in relation to adiponectin isoforms, body weight, and measures of insulin resistance, when assessed as both absolute values and incremental changes.

Discussion

Recent research has demonstrated that 25(OH)Vit D modulates the secretion of adipokines such as leptin and adiponectin *(Walker 2014, Abbas 2017)*. In particular, the most bioactive form of adiponectin, HMW-A, was found to be down-regulated in obese children with VDD, and to increase following cholecalciferol supplementation *(Walker 2014)*. Current results suggest that oral administration of a high dose of cholecalciferol to obese adults with VDD did not significantly impact total adiponectin concentrations but promoted a selective increase of the HMW-A oligomer, which was independent of initial body weight, insulin resistance, and amount of body weight lost on diet. Because HMW-A is a putative modulator of insulin homeostasis, and its changes were specific to cholecalciferol treatment, it seems conceivable that cholecalciferol may intervene on molecular intermediates of glucose homeostasis and influence insulin sensitivity.

Obesity is a global health problem and an important risk factor for diabetes, cancer, heart disease, and hypertension. Obesity and the metabolic syndrome have a high impact on the current epidemic of VDD *(Holick 2005)*. Owing to its pleiotropic actions, supplementation with vitamin D3 and its congeners is hypothesized to act beneficially on several markers of chronic diseases *(Autier 2014)*. It has been shown that serum 25(OH)Vit D concentrations are inversely correlated with body mass index (BMI), fat mass, or percentage of body fat, as well as waist circumference *(Ding 2012)*. It is not clear whether this association is due to increased storage in the adipose tissue, sedentary lifestyle, low sunlight exposure, true vitamin D deficiency, genetic changes in vitamin D metabolism, or unknown factors *(Holick 2005)*. Interventional studies investigating 25(OH)Vit D action on weight gain *(Caan 2007)*, glucose abnormalities *(Pittas 2007)*, and lipid profiles *(Major 2007)* failed to provide reproducible results. However, problems remain with the daily amount of vitamin D3 supplementation, which was in these studies lower than the vitamin D intake considered to be appropriate for adults *(Vieth 2007, Aloia 2008)*. Current evidence collected from randomized trials does not support an effect for

short-term vitamin D supplementation on glucose control in a heterogeneous population with type 2 diabetes, whereas a favorable effect of vitamin D on fasting glucose was seen in patients with poorly controlled diabetes *(Krul-Poel 2017)*. In overweight and obese subjects, high dose vitamin D3 supplementation was also reported to amplify the beneficial effect of weight loss on traditional and non-traditional cardiovascular disease risk markers, independent of changes in body weight, fat mass, and abdominal fat mass *(Zittermann 2009)*. In agreement with previous findings *(Cipriani 2010, Camozzi 2016)*, our study observed that administration of a high cholecalciferol dose in VDD obese subjects caused a prompt and sustained increase of 25(OH)Vit D levels for the 28-day study period. The main finding of our study was the statistically significant incremental modification of HMW-A expression detected by western immunoblot after cholecalciferol administration but not after placebo. This increment appeared slightly delayed as compared to the peak of 25(OH)Vit D levels in the circulation, thus the peak 25(OH)Vit D levels occurred after 7 days while HMW adiponectin peaked after 14 days post-administration, thus suggesting a possible genomic effect. By two-way ANOVA, however, there was no time × treatment interaction, likely due to the small sample. We found a similar incremental effect exerted by cholecalciferol and placebo on MMW-A and LMW-A profiles, which was likely consequent to the diet-induced weight loss. While it should be noted that the placebo group tended to have slightly milder baseline measures of severe obesity and metabolic dysfunction compared to the vitamin D group, these differences were not statistically significant and, thus, we hypothesize that this divergence played a negligible effect on the main findings of this study. In support of our findings obtained by WIB on cholecalciferol and placebo, previous studies showed that supplementation with vitamin D3 at therapeutic doses did not impact total adiponectin concentrations *(Dinca 2016)*, but it selectively increased HMW-A levels in obese adolescents treated for 1 year with cholecalciferol *(Walker 2014)*. While our study and other studies failed to record associations between short-term increases of HMW-A and improvements in insulin sensitivity *(Mai 2014, Polak 2007)*, long-term studies documented a significant correlation between modifications of HMW-A and insulin sensitivity *(Gueugnon 2012, Martos-Moren 2010, Swarbrick 2006)*. Based on our study and long-term clinical data, we hypothesize an insulin-sensitizing effect of vitamin D supplementation through HWM-A.

At odds with results obtained by WIB, our investigation on multimeric adiponectin quantitated by ELISA failed to capture modifications of HWM-A related to cholecalciferol administration, nor did it detect the increases of MMW-A and LMW-A identified by WIB in both groups. There are no comparative data in the literature that fully address this issue, and it cannot be excluded that it may depend on the performance of the methodologies herein used. We, thus, speculate that this discrepancy may reflect intrinsic differences between qualitative/semiquantitative (western immunoblot) and quantitative (ELISA) analyses. This incongruence has been previously noted in a study by Polack and colleagues, where an increase in all three types of adiponectin multimeric complexes was identified by western immunoblot but it was not accompanied by significant changes in total plasma adiponectin levels measured by ELISA *(Polak 2007)*. Finally, such a discrepancy could also reflect different binding capacities of the different monoclonal antibodies used by the two methods.

From a mechanistic viewpoint, adipose tissue is indeed a major site of vitamin D storage, and both the VDR and vitamin D metabolizing enzymes are expressed in adipocytes. Partly contradictory results exist on the adipogenic effect of 1,25(OH)2Vit D *(Abbas 2017)*, which was shown to inhibit adipogenesis in 3T3-L1 mouse preadipocyte cell lines *(Blumberg 2006)*, while promoting adipogenesis and increasing adiponectin expression in primary mouse and human subcutaneous preadipocytes *(Nimitphong 2012)*. Similarly, treatment with 1,25(OH)2Vit D in 3T3-L1 cells was found to stimulate the synthesis of adiponectin and its multimeric forms *(Dinca 2016)*. Moreover, a key regulator of folding and assembly of adiponectin, the endoplasmatic reticulum (ER) chaperon DsbA-L, was found to be upregulated by 1,25(OH)2Vit D, which suggests that vitamin D regulation of adiponectin may involve post-transcriptional mechanism/s *(Walker 2014)*. Alternatively, no effect of 1,25(OH)2Vit D on adiponectin expression was found in human adipocyte cultures *(Sun 2007)*, suggesting that the effect of vitamin D on adiponectin secretion is on open field and warrants further studies.

Our findings also indicate that, in the short term, vitamin D3 supplementation had no additional effect on the magnitude of weight loss compared to placebo, which agrees with previous evidence that vitamin D3 supplementation does not promote improvements in the degree of obesity *(Mallard 2016)*. On the other hand, we also observed that weight loss obtained without Vitamin D3 supplementation did not modify

circulating 25(OH)Vit D levels, which agrees with the inference that loss of adipose tissue does not promote the release of 25(OH)Vit D release from fat into the bloodstream *(Piccolo 2013)*. Although vitamin D3 supplementation does not act synergistically on adiponectin and leptin levels *(Duggan 2015)*, we found a reduction of the leptin/adiponectin ratio in both groups, while a reduction of the leptin/HMW-A ratio was only obtained in the VD group. Based on the predictive role of these measures on metabolic health *(Satoh 2004, Kotani 2005, Inoue 2005, Inoue 2006)* and response to anti-diabetic therapy *(Lilja 2012)*, such a result may add strength to the metabolic role of vitamin D3 administration, independent of changes in body weight. This suggestion is substantiated in studies on obese adolescents, where a 6-month cholecalciferol supplementation (4000 IU/day) significantly decreased the leptin/adiponectin ratio in the absence of changes in BMI or waist circumference *(Belenchia 2013)*.

As limitations of our study, we acknowledge that the small sample size may hamper the clinical significance of our observations. However, the randomized placebo-controlled design of the study is a potential point of strength in addition to the tight monitoring of study effects during the inpatient dieting program. As all participants received a weight loss intervention leading to similar outcomes between groups, we could discriminate independent effects of vitamin D3 supplementation in our experimental conditions. Indeed, the duration of the study and the obese sample does not allow for insights to be gained on the long-term effects vitamin D3 supplementation, or extend our findings to lean populations and different clinical settings. Finally, our study cannot provide insights on translational and post-translational mechanisms involved in modifications of adiponectin oligomers.

Conclusions

In conclusion, we found that an acute dose of cholecalciferol in obese VDD subjects promoted changes of HMW-A independent of changes in body weight and insulin resistance. We observed a decrease in the ratio between leptin and adiponectin or HMW-A. These results could not be confirmed by immunoassay procedures. Further studies are needed to clarify the mechanisms underlying these effects.

Tables

Table 1. Summary of anthropometric and metabolic data in the two populations obtained at baseline and at the end of the study.

	Vitamin D Group (n=12)			Placebo Group (n=12)			p Value		
	Baseline	At the end of the study	Δ (%)	Baseline	At the end of the study	Δ (%)	Time	Treatment	Time x Treatment
Male/Females	6/6	-	-	7/5	-	-	-	-	-
Age (years)	38±2.4	-	-	37±3.0	-	-	-	-	-
BMI (kg/m^2)	42.7±1.3	40.1±1.2**	-6.0±0.7	39.8±0.9	37.6±0.7**	-5.5±0.5	<0.0001	0.07	0.41
Body Weight (kg)	115.9±4.4	109.2±4.9**	-5.8±0.4	112.7±4.7	106.4±4.3**	-5.5±0.5	<0.0001	0.64	0.65
Fat mass (%)	44.8±1.6	-	-	40.4±1.6	-	-	-	-	-
Free fat mass (kg)	63.4±2.9	-	-	66.7±3.3	-	-	-	-	-
Waist (cm)	126.2±3.5	-	-	119.0±4.0	-	-	-	-	-
Glucose (mg/dL)	97.0±7.1	90.2±3.1	-7.1±4.1	88.5±3.2	83.0±1.6*	-5.5±2.1	0.07	0.45	0.10
Insulin (mU/L)	13.9±2.5	14.5±1.7	5.9±12.7	17.5±2.2	14.1±1.3	-18.5±8.1	0.06	0.17	0.30
HbA1c (%)	5.5±0.1	5.7±0.3	0.7±3.2	5.6±0.6	5.3±0.2*	-4.7±1.3	0.21	0.53	0.07
HOMA-IR	3.9±0.6	3.3±0.4	-3.2±11.3	3.7±0.5	3.0±0.2	-9.7±8.3	0.11	0.67	0.99
25(OH) Vit D (ng/mL)	14.2±1.9	35.0±3.2$^{\#,**}$	191.0±39.6	14.5±1.9	14.8±1.6	7.2±6.3	<0.0001	<0.0001	<0.0001
Total adiponectin (µg/mL)	3.6±0.5	3.5±0.4	0.2±5.9	4.0±1.1	3.9±1.0	1.9±9.1	0.39	0.75	0.89
Leptin (ng/mL)	44.1±7.2	31.6±5.9*	-27.3±5.4	41.1±4.6	9.8±4.1**	-29.9±4.0	<0.0001	0.75	0.72
Leptin/Adiponectin	15.9±4.3	10.5±2.7*	-23.6±5.4	14.8±2.5	10.3±1.6	-21.2±12.9	<0.01	0.86	0.73
Leptin/HMW-A	49.1±12.0	24.7±4.2*	-26.8±10.6	37.4±7.4	35.5±9.9	-6.9±12.8	<0.05	0.96	<0.05

Data are presented as mean SEM. p-values refer to the effect of time, treatment, and time treatment assessed by two-way ANOVA. For significance: a paired t-test was performed in each group between baseline and study-end assessment ($^{**}p < 0.001$, $^{*}p < 0.05$); an unpaired t-test was performed between the two groups at baseline and at the end of the study ($^{\#}p < 0.01$). For abbreviations: BMI, Body Mass Index; HbA1c, Glycated Haemoglobin; HOMA-IR, Homeostatic Model of Insulin resistance; 25(OH)Vit D, 25-hydroxycholecalciferol; HMW-A, High Molecular Weight Adiponectin; Δ (%), percent delta value (listed as mean ± SEM).

Table 2. Biochemical evaluation of multimeric adiponectin by ELISA.

	Vitamin D Group (n=12)			Placebo Group (n=12)			p Value		
	Baseline	14 Days	28 Days	Baseline	14 Days	28 Days	Time	Treatment	Time x Treatment
Total Adiponectin (µg/mL)	3.6±0.5	3.7±0.4	3.5±0.4	4.0±1.1	3.9±0.9	3.8±1.0	0.94	0.51	0.99
HMW-A (µg/mL)	1.6±0.4	1.6±0.2	1.5±0.2	2.1±0.8	1.9±0.5	1.8±0.7	0.92	0.38	0.97
MMW-A (µg/mL)	0.7±0.1	0.6±0.1	0.7±0.1	0.5±0.2	0.4±0.2	0.8±0.2	0.63	0.93	0.87
LMW-A (µg/mL)	1.5±0.2	1.5±0.2	1.4±0.2	1.5±0.3	1.8±0.4	1.5±0.4	0.64	0.51	0.74

Figures

Figure 1. Effect of cholecalciferol or placebo administration on serum 25(OH)Vit D levels. Data are presented as mean ± SEM. For significance: *p<0.001 vs. baseline within group, as calculated by repeated measures one-way ANOVA; ‡p<0.0001 between groups by unpaired t-test. Two-way ANOVA was also performed to test the effect of time, treatment, and time × treatment interaction, and results are summarized in the text and Table 1.

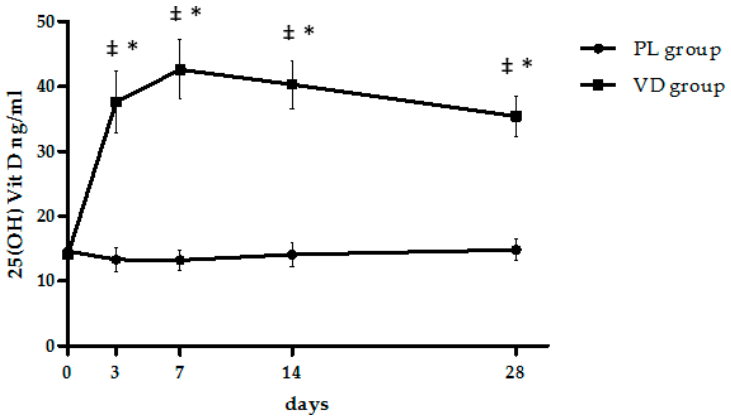
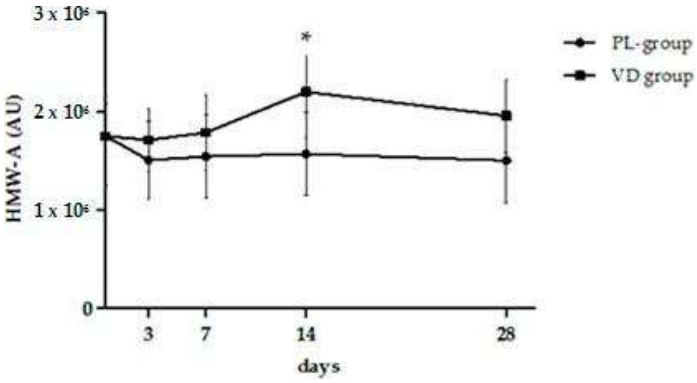
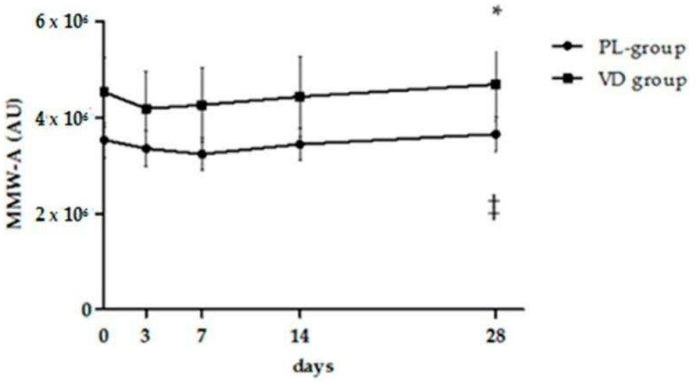


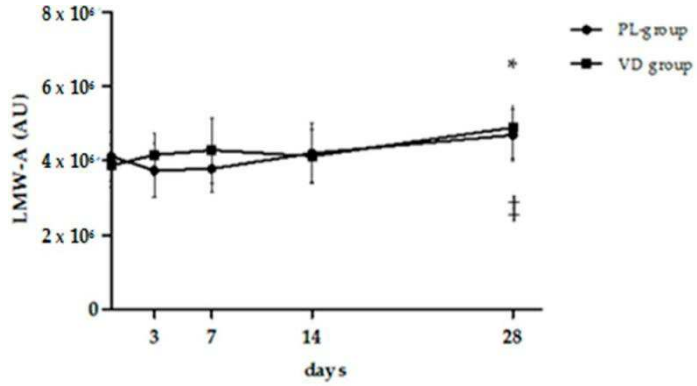
Figure 2. Effect of cholecalciferol or placebo administration on serum adiponectin oligomer expression levels measured by western immunoblot. Data are presented as mean ± SEM. Values are presented as arbitrary units (AU) normalized to total protein determined by Ponceau S staining.



(a) High molecular weight adiponectin (HMW-A): for significance in the VD group: *p<0.05 vs. baseline, 3 and 7 days after treatment (repeated measures one-way ANOVA), no difference between groups by unpaired t-test



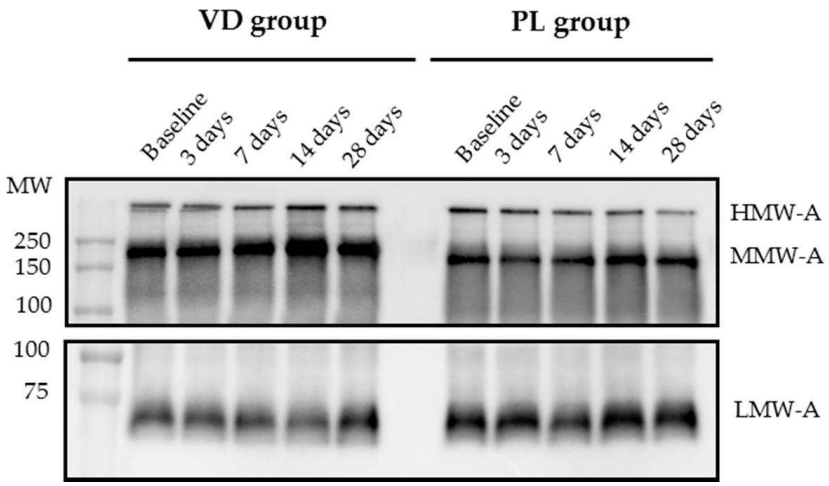
(b) medium molecular weight adiponectin (MMW-A): for significance: *p<0.05 vs. 3 days in the VD group; ‡p<0.05 vs. 7 days in PL group (repeated measures one-way ANOVA), no difference between groups by unpaired t-test



(c) low molecular weight adiponectin (LMW-A): for significance: *p<0.05 vs. 3, 7, and 14 days in the VD group; ‡p<0.05 vs. 3, 7 days in the PL group (repeated measures one-way ANOVA), no difference between groups by unpaired t-test.

Two-way ANOVA was also performed to test the effect of time, treatment, and time × treatment interaction, and results are summarized in the text.

Figure 3. Representative immunoblots of high (HMW-A), medium, (MMW-A), and low molecular weight adiponectin (LMW-A) changes obtained at baseline and after 3, 7, 14, and 28 days following cholecalciferol or placebo administration in obese subjects. Representative western immunoblot (WIB) analyses under non-reduced conditions are displayed. HMW-A and MMW-A were analyzed following P1 digestion, while LMW-A was analyzed directly (for description see text). MW = molecular weight.



Serum uric acid potentially links metabolic health to measures of fuel use in lean and obese individuals

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Abstract

Purpose. Uric acid (UA) is a byproduct of the high-energy purine metabolism that is conventionally regarded as a marker of cardio-metabolic impairment. Its potential relationship with energy homeostasis is indeterminate.

Methods. We performed a cross-sectional study on 121 otherwise healthy obese and 99 sex- and-age-matched lean subjects to analyze UA levels in relation to metabolic health, inflammatory markers, respiratory quotient (RQ) and resting energy expenditure (REE) assessed by indirect calorimetry, fat mass (%FM) and fat-free mass (FFM) measured by bioimpedance analysis.

Results. Obese and lean subjects differed as expected in BMI, glucolipid homeostasis, leptin and insulin levels, inflammatory markers, %FM and FFM ($p<0.001$ for all). Obese had higher UA levels ($p<0.001$) and higher rates of hyperuricaemia (40.5% vs 3.0%, $p<0.0001$) than controls. Indirect calorimetry revealed that obesity increased REE and decreased RQ significantly ($p<0.001$). Beyond expected metabolic correlates, correlation analysis both in individual and merged datasets revealed that UA was associated negatively with RQ and positively with REE ($p<0.0001$ for both). In multivariable regression analysis, significant independent predictors of UA were REE, RQ, BMI, and sex. When BMI was replaced by measures of body composition, FFM emerged as only significant predictor of serum UA ($p<0.0001$).

Conclusions. The associative link between serum UA and REE reveals a potential role for UA in managing obesity energy homeostasis.

Background

Most mammals excrete UA, a byproduct of purine degradation *(Wu 1989)*, upon conversion to soluble allantoin by the liver enzyme urate oxidase (uricase) *(Hayashi 2002)*. In humans, the lack of uricase causes UA accumulation *(Richette 2010)* and promotes hyperuricemia under the influence of inappropriate dietary and life-style factors *(Choi 2004)*. Upon urate super-saturation, crystalline monosodium urate becomes susceptible to precipitation leading to potentially serious complications, such as gout and kidney disease *(Doherty 2009)*. Non-crystalline soluble component of UA possesses a unique amphipathic action *(Sautin 2008)* and exerts pro-oxidant effects in vascular cells mediated by lipid oxidation, which can lead to endothelial dysfunction and cardiovascular disease *(Kanellis 2005, Sánchez-Lozada 2006)*. On the other hand, soluble UA acts as a powerful antioxidant that scavenges oxygen singlets and oxygen radicals, peroxynitrite, and chelates transition metals, thus protecting the cell membrane and DNA from oxidative damage *(So 2010, Kuzkaya 2005, Ames 1981)*.

Physiologically, UA levels are higher in men than women, increase with age and menopause, and change under the influence of antiuricosuric drugs, such as thiazide diuretics *(Mikuls 2006, Rho 2011)*. With the gradual progression of obesity and diabetes mellitus, UA accumulation has been increasingly referred to as a proxy of the so-called metabolic syndrome *(Yang 2012, Modan 1987)*, due to its robust association with obesity, visceral adipose tissue (VAT) accumulation, insulin resistance, systemic inflammation and muscle loss *(Onat 2006, Ogbera 2010, Matsuura 1998, Tamba 2008, Hikita 2007, Huang 2013)*. Obesity-associated hyperuricaemia is prompted by a number of mechanisms, including the following: overexpression of xanthine oxidoreductase in adipose tissue, which results in enhanced purine catabolism and increased production of UA *(Tsushima 2013)*; hyperinsulinaemia and insulin resistance, which decrease renal UA excretion *(Facchini 1991)*; 3) VAT accumulation, which promotes fatty acid (FA) output in the portal vein *(Chen 2007, Clausen 1998)* and, as a result of the excessive metabolic outflow, promotes de novo synthesis of purine from NADPH in the pentose phosphate pathway and increased UA production *(Matsuura 1998, Fabregat 1987, Matsubara 1989)*; increased consumption of purine-rich food, especially meat and to a lesser extent fish, alcoholic drinks and soft drinks rich in fructose *(Wallace 2004, Choi 2008)*. Blood UA

reflects the balance between purine dietary uptake, production and catabolism of purines, and production and excretion of UA. Although purine turnover is relatively constant at 300-400 mg/day, dietary factors accelerate purine nucleotide depletion and rates of de novo purine synthesis, thus potentiating UA production *(Choi 2010)*. De novo purine biosynthesis is a high-energy process that leads to the conversion of phosphoribosyl pyrophosphate (PRPP) to inosine monophosphate (IMP) through 10 enzymatic activities and consumption of 6 ATP molecules, whereas only one ATP molecule is required for the purine salvage pathway consumption *(Buchanan 1994, Seegmiller 1975, Becker 1993)*. Thus, production and excretion of UA in humans reflects energy utilization and could act as a surrogate measure of fuel utilization and energy homeostasis. On this basis, we sought to investigate if UA could reflect energy storage and explored the relationship between circulating UA and the components of energy metabolism, e.g. respiratory quotient (RQ) and resting energy expenditure (REE), in a cross-sectional analysis in lean and obese adult individuals.

Methods

Patients

This study enrolled 121 obese patients (52 males; age 18-58 yr; body mass index (BMI), 43.1±7.1 kg/m2) referred to our Institution for work-up and rehabilitation of obesity and its comorbidities, and 99 matched lean subjects (34 males; age 19-53 yr; BMI, 22.5±2.5 kg/m2). All women were premenopausal, as assessed by personal history of regular menses. Exclusion criteria for both groups included medications interfering with UA levels and hypertriglyceridaemia. Patients were also excluded if suffering from autoimmune diseases, tumors, polycythemia, hemolysis, diabetes, liver or kidney diseases, smoke and alcohol consumption. The investigation was approved by the ad hoc Ethical Research Committee of Istituto Auxologico Italiano, functioning according to the 3rd edition of the Guidelines on the Practice of Ethical Committees in Medical Research. Written consent was obtained from all patients and controls, after full explanation of the purpose and nature of the study.

Body measurements

All subjects underwent body measurements wearing light underwear, in fasting conditions after voiding. Weight and height were measured to the nearest 0.1 kg and 0.1 cm, respectively, using standard methods. BMI was expressed as body mass (kg)/height $(m)^2$. Obesity was defined for any BMI over 30 kg/m^2.

The respiratory quotient (RQ; VO2/VCO2) and resting energy expenditure (REE; kcal/24 h) were determined in a thermoregulated room (22-24°C) by computed open-circuit indirect calorimetry, measuring resting oxygen uptake and resting carbone dioxide production by a ventilated canopy (Sensormedics, Milan, Italy) at 1-min intervals for 30 minutes and expressed as a 24-h value. The test consists of making each patient lie down relaxed on a comfortable armchair, with the head under a transparent hood connected to a pump, which applies an adjustable ventilation through it. Exhaled gas dilutes with the fresh air ventilated under the hood and a sample of this mixture is conveyed to the analysers, through a capillary tube and analysed. Ambient and diluted fractions of O2 and CO2 are measured for a known ventilation rate, and O2 consumption (VO2) and CO2 production (VCO2) are determined. Energy expenditure was calculated according to the Weir equation *(Weir 1949)*: EE = 5.68 VO2 + 1.59 VCO2 – 2.17 Nu. As short-term urinary collections to assess total nitrogen excretion (Nu) may not be representative of the protein oxidized during the measurement itself, they were not be obtained in this study, and assumed to be 13g/24h *(Frayn 1983)*. The predicted REE (pREE) was calculated by the Harris-Benedict formula and allowed to test for metabolic efficiency, calculated as the ratio between measured/predicted REE values, as previously reported *(Marzullo 2004)*.

Percent fat body mass (FM) and lean body mass (FFM) were determined by bioelectrical impedance analysis (BIA) (model BIA 101/S Akern, Florence, Italy). Patients with fluid overload according to vectorial analysis were excluded to minimize errors in estimating FM and FFM in severe obesity *(Piccoli 1998)*.

Assays

Blood samples were drawn under fasting conditions (8-12 h). For each participant was required to avoid physical activity, caffeine and dietary supplements 24 h prior to testing.

Blood levels of UA, glucose, total cholesterol, high-density (HDL) and low-density (LDL) lipoprotein cholesterol and triglycerides were measured by enzymatic methods (Roche Diagnostics, Mannheim, Germany). Serum insulin levels were measured using a Cobas Integra 800 Autoanalyzer (Roche Diagnostics, Indianapolis, IN, USA). Ultrasensitive C-reactive protein was measured by CRP (latex) HS Roche kit.

Insulin resistance was calculated by the homeostasis model of assessment-insulin resistance (HOMA-IR) approach, calculated as insulin (microunits per milliliter) x blood glucose (millimoles per liter)/22.5. Serum leptin levels were measured by enzyme-linked immunosorbent assay (Human Leptin ELISA, Mediagnost, Reutlingen, Germany). The minimum detectable concentration was 0.2 ng/mL. The intra-assay and inter-assay coefficients of variation were <10%.

Statistical analysis

Statistical analysis was performed using SPSS version 21 (Somers, NY, USA). Values are expressed as means ± standard deviation (SD). Continuous variables were log transformed if distribution resulted skewed by the Shapiro-Wilk normality test. Comparative analyses within and between groups were performed by Mann-Whitney U test or two-tailed unpaired Student's t test after Welch's correction, when appropriate. The simple chi-square test was used for associations between categorical variables. Spearman's correlation analysis was used to identify significant associations between variables of interest. Stepwise multivariable regression analysis was used to evaluate the independent association of variations in UA with metabolic, anthropometric or biochemical parameters. β coefficients and related significance values obtained from the models are reported. $P<0.05$ was considered as statistically significant.

Results

A summary of anthropometric, metabolic an biochemical data is reported in Table 1 (pag. 59). As predicted, obese and lean subjects differed for measures of adiposity, glucose and lipid metabolism, inflammatory markers, and leptin levels. Compared to lean individuals, obese patients had higher REE and lower RQ values. Obese subjects showed higher UA

levels, and hyperuricemia was documented in 40.5% of obese and 3.0% of lean subjects ($\chi 2=40.6$, $p<0.0001$). Correlation analysis between UA and variables of interest in the population as a whole and in the two groups separately (Table 2, pag. 60) showed an expected inverse correlation between UA and female gender, while a positive association related UA to impaired glucolipid metabolism, inflammatory markers, body composition and anthropometric variables related to visceral adiposity. A strong correlation was observed between serum UA and FFM in separate and merged groups. All correlations obtained in the entire dataset were lost after controlling for gender, age and BMI. When serum UA was plotted against the components of energy homeostasis, RQ and REE, divergent associations emerged. As such, an overall negative association related serum UA to RQ values in merged dataset, while it fail to reach statistical significance in separate groups. This association persisted after controlling for gender, age and BMI. Oppositely, UA levels were positively associated with REE (Figure 1, pag. 61) and this association persisted when REE was normalized by FFM ($rho=0.13$, $p<0.05$). After controlling for BMI, age and gender as individual covariates, the association between REE and UA remained significant, whereas it was abrogated when these variables were included collectively in analysis. Alternatively, the correlation between UA and either the predicted REE or the measured/predicted REE ratio, a surrogate measure of metabolic efficiency, was null.

Based on correlations obtained between groups, a number of stepwise regression models were performed between UA and metabolic, anthropometric and body composition parameters. With the purpose of avoiding collinearity, a general model was created that comprised either BMI or FFM and %FM, either RQ or REE, as well as sex (0=males, 1=females), HOMA-IR, HDL and triglycerides. When BMI and RQ were introduced with the other variables, the only predictors were BMI ($\beta=0.60$, $p<0.0001$), sex ($\beta=-0.37$, $p<0.0001$) and RQ ($\beta=0.11$, $p<0.05$) with an adjusted R2 value of 0.58. In a model with REE, this was excluded and the only predictors were BMI ($\beta=0.59$, $p<0.0001$) and sex ($\beta=-0.37$, $p<0.0001$) with an R2 of 0.58. Replacement of BMI with FFM and %FM in a model that had RQ and the other variables showed that predictors of UA were FFM ($\beta=0.34$, $p<0.0001$), %FM ($\beta=0.39$, $p<0.0001$), sex ($\beta=-0.32$, $p<0.0001$) and RQ ($\beta=0.10$, $p<0.05$) with a R2 value of 0.56. REE instead of RQ had no statistical value, with the only predictors being FFM ($\beta=0.29$, $p<0.0001$), %FM ($\beta=0.36$, $p<0.0001$), sex ($\beta=-0.32$, $p<0.0001$) and triglycerides ($\beta=0.11$, $p<0.05$), with a R2 value of 0.55. Interestingly,

analysis of the effect of FFM and %FM on UA showed that FFM was a stronger predictor of UA (β=0.61, p<0.0001) than %FM (β=0.24, p<0.0001) and the R2 value was 0.51.

Discussion

The results of our study show that serum UA, in addition to reflect metabolic derangement, is a surrogate index of the resting energy state in lean and obese subjects. We observed that UA levels were inversely associated with values of RQ and increased with increasing values of REE. Multivariate analysis found that UA was an independent predictor of REE. This finding suggests that UA may be involved in the regulation of substrate availability and energy stores.

UA is a recognized marker of metabolic derangement widely used for its ability to predict the risk of cardio-metabolic morbidity *(Abdelmalek 2012, Yamashita 1986)*. Dietary excess, fat accumulation and insulin resistance are all known causes of increased UA, which enhance the risk of gout and kidney disease. Among the NHANES participants, a 1.3–1.5 times greater than normal prevalence of gout was found in overweight individuals after adjustment for serum UA, while the prevalence ratio was 1.8 for class I obesity and 2.2–2.4 for class II or III obesity *(Juraschek 2013)*. Epidemiologic studies have repeatedly manifested the ability of serum UA to associate with cardio-metabolic disorders, e.g. hypertension, coronary artery disease, cerebrovascular disease, and kidney disease, not just if overt hyperuricemia occurs but also when serum UA approaches the high-normal range *(Feig 2008)*. Such unfavourable link is herein confirmed by the clustering of UA levels with variables related to obesity and derived indexes, i.e. metabolic setting, fatty body composition and abdominal adiposity. One novel finding was a divergent association observed between UA levels and components of fuel use. Our obese cohort had lower RQ values than lean controls, a finding that originates from the effects of the fat mass on the rate of FA oxidation *(Rossow 2014)*. RQ values were linearly and inversely associated with UA levels, and this relationship was not influenced by hyperuricaemia, i.e. subjects with hyperuricaemia did not show lower RQ levels that those without. However, the correlation lost significance disappeared in separate group, likely due to the narrow regression of RQ values. RQ, which represents the moles of carbon evolved per mole of oxygen consumed, reflects macronutrients metabolism *(Widmaier 2016)* and RQ values of

0.7, 0.8 and 1.0 are conventionally used in humans to identify the prominent oxidation of lipids, proteins and carbohydrates, respectively *(Widmaier 2016)*. Previous evidence has showed that UA production and lipid metabolism are related in gout *(Matsubara 1989)*, and obese adipose tissue is characterized by active FA synthesis *(Spiegelman 1996)* and de novo purine synthesis through the activation of the pentose phosphate pathway *(Leyva 1998)*, which could contribute to increase UA synthesis and excretion *(Becker 2002)*. On the other hand, also lipolysis is up-regulated in obese adipose tissue *(Greenberg 2011)* and a high content of FA may flow into the liver directly through the portal vein in subjects with visceral fat accumulation, thereby accelerating TG synthesis in the liver and ultimately promoting hypertriglyceridemia *(Bjmtorp 1990)*. According to this flow, we too observed that UA associated with plasma TG and abdominal adiposity. Hence, it can be hypothesized that UA could signal the rate of FA oxidation, e.g. the switch from glycolysis to lipolysis.

Another result worth of mention was the positive association between serum UA and REE values, which was evident in single-group and whole-group analysis. At the multivariable regression analysis, REE was excluded from the equation, suggesting that anthropometric variables maintain greater control of UA levels. It remains difficult to conceptualize on these results based on the lack of data in the literature, with the exception of an abstract reporting on a correlation between serum UA and REE in a study on obese women *(Kerksick 2009)*. It has long been shown that energy expenditure varies among people independent of body size and composition, and that skeletal muscle metabolism is the main determinant of metabolic rate, such that differences in resting muscle metabolism can produce differences in metabolic rates among individuals *(Zurlo 1990, Bosy-Westphal 2004)*. While it can be argued that the link between UA and REE could depend on the effect of fat-free mass between obese and lean individuals, we found the same correlation when REE was adjusted for FFM, suggesting that FFM was not involved in the association. It should be stated, however, that body composition was herein measured by BIA, which per se is less informative than DXA due to the potential effect of fluid overload. Literature data suggests that UA, as a marker of cell metabolism, can increase in conditions associated with high REE such as hyperthyroidism *(Sato 1995)*, HIV-related infection *(Walker 2006, Hommes 1990)*, and hypoxia from chronic obstructive pulmonary disease or sleep apnea *(Creutzberg 1998, Garcia-Pachon 2007)*. As we did not assess sleep

apnea in our obese patients, this issue remains open. When adipose tissue accumulation is accounted for, there is evidence that adipocyte differentiation upregulates intracellular accumulation of triglyceride and UA secretion, suggesting that the activation of the pentose phosphate pathway related to FA synthesis is associated with the activity of xanthine oxidoreductase and production of UA *(Tsushima 2013)*. Purines are endogenous modulators of energy metabolism and signal transduction, and play important roles in the physiology of platelets, muscles and neurotransmission *(Maiuolo 2016)*. De novo purine biosynthesis is a high-energy event that leads to the conversion of phosphoribosyl pyrophosphate (PRPP) to inosine monophosphate (IMP) through a process requiring 10 enzymatic activities and 6 ATP molecules, while only one ATP molecule is required for the purine salvage pathway *(Buchanan 1994, Seegmiller 1975)*. Bearing in mind that UA production provides an indirect estimate of ATP degradation, and because any increase in energy expenditure for a given amount of substrate requires either increased ATP hydrolysis or decreased ATP synthesis *(Ricquier 2005)*, UA could be involved in signalling ATP availability, hence the energy state to other organs or apparatuses. As independent factor, UA has been already shown to mediate its effects by inducing oxidative stress, inflammation, endothelial dysfunction and activation of the renin angiotensin aldosterone system, which play a role in the pathogenesis of cardiovascular disease *(Kanbay 2013)*.

Our study has some important limitations that should be mentioned. Firstly, the retrospective, cross-sectional design of the study limits our ability to determine excretion of UA in the urine, that could allow a comparison between the excretion rate and circulating UA levels. This could possibly explain the retention of UA in obese vs lean individuals. Secondly, we did not measure ATP levels, which could allow us to discuss the role of UA as an indirect measurement of ATP degradation. Finally, we cannot assess a "one-to-one" matching of partecipants analysis because of the retrospective design of our study. Notwithstanding these limitations, this study has important strengths, including the accurate analysis of REE, the inclusion of pre-menopausal women and the evaluation multiple anthropometric and biochemical parameters.

Conclusions

In summary, UA could be one of those factors that signal the systemic oxidative phosphorylation and are able to fine tune the energy state required for cell differentiation and metabolic requirements. Further studies are warranted to clarify if the relationship between UA and REE reflects a functional link or acts as an adaptive physiological response to abnormal substrates oxidation.

Tables

Table 1. Summary of anthropometric, metabolic and biochemical parameters in lean subjects and obese patients. Significance between groups was calculated by Mann-Whitney test for continuous variables or the chi-square for categorical variables.

Parameters	Obese subjects	Lean subjects	P
Males/Females	52/69	34/65	0.2
Age (years)	35.1±9.1	35.3±7.9	0.8
BMI (Kg/m^2)	43.1±7.1	22.5±2.5	<0.001
WC (cm)	121.0±26.5	63.0±10.8	<0.001
FM (%)	45.0±6.7	24.9±6.4	<0.001
FFM (kg)	66.7±17.7	47.6±8.5	<0.001
RQ (VO_2/VCO_2)	0.84±0.08	0.90±0.12	<0.001
REE (Kcal/24 h)	2054±432	1577±255	<0.001
pREE (Kcal/24 h)	2119±471	1469±203	<0.001
REE/pREE	0.98±0.10	1.08±0.13	<0.001
UA (mg/dL)	6.49±1.51	4.32±1.19	<0.001
CHO (mg/dL)	200.9±35.2	198.6±36.7	0.6
HDL CHO (mg/dL)	46.9±15.7	63.8±15.9	<0.001
LDL CHO (mg/dL)	135.4±32.6	131.6±83.8	0.6
TG (mg/dL)	154.8±78.5	93.6±43.2	<0.001
Insulin (mIU/mL)	14.2±7.3	9.7±8.8	<0.001
Glucose (mg/dL)	84.6±13.6	86.0±14.5	0.4
HOMA-IR	3.05±1.78	1.94±1.42	<0.001
CRP (mg/dL)	0.93±0.96	0.19±0.32	<0.001
Fibrinogen (mg/dL)	407.7±66.9	305.5±67.8	<0.001
Leptin (mg/mL)	33.92±15.18	8.95±7.83	<0.001

For abbreviations: BMI, body mass index; WC, waist circumference; TBW, total body water; FM, fat mass; FFM, free fat mass; RQ, Respiratory Quotient; REE, resting energy expenditure; pREE, predicted resting energy expenditure; UA, uric acid; CHO, total cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; TG, triglycerides; HOMA-IR, homeostatic model of insulin resistance; CRP, C-reactive protein.

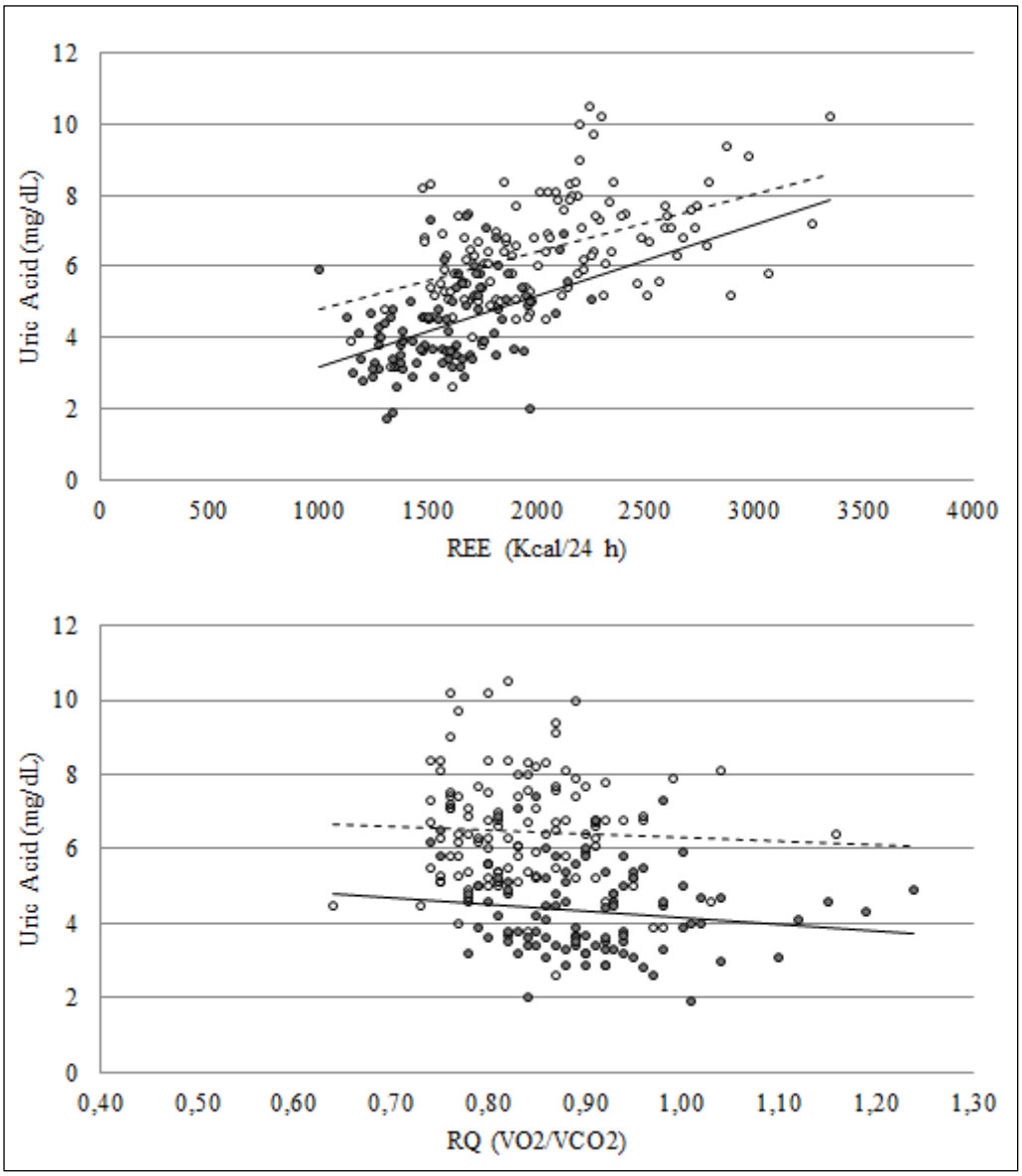
Table 2. Spearman's correlation analysis between serum UA levels and variables of interest in merged groups (whole), obese patients and lean subjects. For significance: a, p<0.05; b, p<0.01; c, p<0.001; d, p<0.0001.

Parameters	Whole population	Obese subjects	Lean controls
Age (years)	-0.07	-0.15	0.04
BMI (Kg/m^2)	**0.64**[d]	**0.25**[b]	**0.38**[d]
WC (cm)	**0.74**[d]	**0.44**[d]	**0,57**[d]
FM (%)	**0.43**[d]	**-0.34**[d]	-0.12
FFM (kg)	**0.73**[d]	**0.50**[d]	**0.60**[d]
RQ (VO_2/VCO_2)	**-0.32**[d]	-0.02	-0.11
REE (Kcal/24 h)	**0.66**[d]	**0.46**[d]	**0.47**[d]
CHO (mg/dL)	0.11	0.14	0.11
HDL CHO (mg/dL)	**-0.54**[d]	**-0.29**[c]	**-0.34**[d]
LDL CHO (mg/dL)	**0.24**[d]	0.14	**0.23**[a]
TG (mg/dL)	**0.43**[d]	**0.27**[b]	0.16
Insulin (mIU/mL)	**0.44**[d]	**0.38**[d]	0.04
Glucose (mg/dL)	0.05	0.05	**0.36**[d]
HOMA-IR	**0.44**[d]	**0.36**[d]	0.11
PCR	**0.47**[d]	0.02	0.06
Fibrinogen (mg/dL)	**0.34**[d]	-0.03	-0.11
Leptin (mg/mL)	**0.36**[d]	**0.23**[a]	**0.39**[d]

For abbreviations: BMI, body mass index; TBW, total body water; FM, fat mass; FFM, free fat mass; RQ, Respiratory Quotient; REE, resting energy expenditure; CHO, total cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; TG, triglycerides; HOMA-IR, homeostatic model of insulin resistance; CRP, C-reactive protein

Figures

Figure 1. Bivariate correlation analysis between serum UA levels and RQ (upper panel) or REE (lower panel) in merged datasets from obese subjects and lean controls. Open circles and dashed line: obese subjects; closed circles and broken line: lean controls.



Plasma oxytocin levels in women are independently affected by obesity and menopause

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Abstract

Objective. In addition to its standard neurohypophyseal functions, oxytocin is under investigation for its role in the control of energy balance. Its relationship with obesity and menopause, a critical stage for bodyweight changes, is unknown.

Methods. This cross-sectional study enrolled 56 subjects with obesity (OB) (28 premenopausal and 28 postmenopausal; medium BMI 46±4.5 kg/m2) and 53 normal-weight (NW) women (27 premenopausal and 26 postmenopausal; medium BMI 23±3 kg/m2). Measurements included oxytocin, and adipokines leptin and adiponectin levels; menopause-related hormones; glucose and lipid homeostasis. Percent fat mass (%FM) and fat-free mass (FFM, kg) were measured by DXA.

Results. Oxytocin levels were significantly lower in OB than NW group both in pre- and postmenopausal conditions ($p<0.001$ for both), with 3 OB women having levels below the assay detection limit. In both study groups, menopause was associated with lower oxytocin compared to premenopausal levels (NW, $p<0.05$; OB, $p=0.005$). Multivariable analysis showed that both menopause and obesity, or their derived measures, acted as predictors of oxytocin levels (BMI, $\beta=-0.51$, $p<0.0001$; menopause, $\beta=-0.28$, $p<0.001$), while their interaction was not significant ($F=0.34$, $p=0.5$).

Conclusions. Both obesity and menopause significantly reduced impair oxytocin levels, but their effects are independent. Our findings may contribute to understanding the hormonal changes involved in weight modifications at menopause.

Background

Body weight gain and consequent onset of associated diseases affect a growing proportion of the adult population and represent one of the most pressing worldwide public health problems *(WHO 2015)*. Modifications in hormonal homeostasis, body composition and bone mass are associated with aging, in particular for women aged 55-65 years *(Zain 2015)*. Obesity is a major risk factor for a variety of chronic conditions *(Stevens 2002)*, and changes in the hormonal milieu at menopause can impact fat partitioning and overall body composition *(Karvonen-Gutierrez 2016)*, critical for the onset of type 2 diabetes mellitus (T2DM) and cardio-metabolic disease *(Lobo 2014)*. While energy imbalance and genetic susceptibility are leading causes for overweight and obesity, the mechanisms underlying weight gain at menopause are not completely understood. Estrogens and the estrogen receptor (ER)-α are essential components of the central network that controls appetite, meal size, food intake and body weight, and they significantly impact neural circuits controlling food intake and energy expenditure *(Schwartz 2000)*. Evidence has accumulated that oxytocin, a neurohypophyseal hormone physiologically implicated in labor *(den Hertog 2001)* and psycho-neural functions *(MacDonald 2010)*, also intervenes in controlling food intake and energy balance in concert with leptin *(Cai 2013)*, although their interplay is not completely understood *(Perello 2013)*. At the central level, oxytocin expression is predominantly located within the hypothalamic parvocellular neurons of the paraventricular nucleus (PVN) and magnocellular neurons of both the PVN and supraoptic nucleus, but small amounts of oxytocin are also synthesized in the gastrointestinal tract in rodents and humans *(Ohlsson 2006, Welch 2009)*. In parallel, the oxytocin receptor is widely expressed in relevant areas of the central nervous system (CNS) involved in food intake *(Schwartz 2000)*, as well as in several peripheral tissues, including adipocytes *(Yi 2015)* and gastrointestinal tract *(Monstein 2004)*. Reduced oxytocin signaling associates with obesity and mice deficient in oxytocin or oxytocin-receptors are prone to develop late-onset obesity *(Camerino 2009, Takayanagi 2008)*. On the other hand, peripheral oxytocin administration to obese rats reduced food intake and bodyweight, while preventing the typical reduction of energy expenditure caused by weight loss *(Morton 2012)*. Recently, intranasal oxytocin in obese

subjects, reduced caloric intake, resulting in a shift from carbohydrate to fat utilization and improving insulin sensitivity *(Lawson 2015)*.

Modifications of circulating oxytocin in relation to pathophysiological events following menopause, is to date only a hypothesis *(Breuil 2014)*, with only a limited number of studies investigating the overall changes of oxytocin levels in patients with obesity and T2DM *(Qian 2014)*, as well as premenopausal women with T1DM *(Kujack 2015)*. To date, oxytocin physiology remains overlooked with menopause, while its contribution in energy homeostasis is a burgeoning area of research with its use having interesting potentials as a therapeutic strategy to treat the metabolic disease. In this study, we aimed to determine the levels of plasma oxytocin and its relationship with biochemical, hormonal and indices of adiposity in menopause of both lean and obese state.

Methods

Study participants

The study included women in premenopausal and postmenopausal stage selected from obese subjects referred to our institution for work-up and rehabilitation for obesity with no known systemic comorbidities. Control women were selected from the hospital staff. All study participants were recruited between January 2011 and September 2015. between January 2014 and September 2015. Diagnosis of physiological menopause was based on clinical criteria in women over 45 years who did not report a period for at least 12 months and were not using hormonal contraception, independent of menopausal symptoms *(Sarri 2015)*. For this study, the age limits were arbitrarily set between 40-50 years for premenopausal and 51-60 years for postmenopausal women. The original samples comprised 120 women equally divided between several over-weight (OB) and normal-weight (NW) women, and complete datasets according to criteria herein used were available for 56 severely OB women (28 pre- and 28 post-menopausal) and 53 NW control women (27 pre and 26 post-menopausal). All women in premenopausal stage reported regular menses and were not taking estrogens or other hormone therapies. Exclusion criteria for both groups included history of menstrual irregularities, previous or current treatment for osteoporosis, use of hormone therapy, menopause age <45 years,

previous major gynecological surgery, autoimmune diseases, thyroid disorders, previously known and/or treated T2DM. Informed consent was obtained from each participant before inclusion into the study. The study was approved by the Clinical Research Ethics Committee of our Institute, according to the Declaration of Helsinki.

Clinical examination and body composition analysis

Height and weight were measured with standardized techniques. BMI was expressed as kilograms per square meter. Participants were told to avoid stressful activities (sports and physical exercise) for at least three days before blood sampling. Information collected by each participant included the following data: age, age at menarche, age at menopause, number of pregnancies, current treatments, past medical history. Dual-energy x-ray absorptiometry (DXA) (GE Lunar Corp. Madison, WI, USA) was used for the assessment of body mass, expressed as lean body mass in kilograms and fat body mass as the percentage of total body mass, as well as for the assessment of the regional percentage of trunk fat mass.

Biochemical parameters

Blood samples were withdrawn from an antecubital vein after 10 hours overnight fast, in follicular phase for premenopausal women. Blood samples were separated by centrifugation after clotting, and aliquots of serum or plasma supernatants were processed for routine measurements and stored at -80°C until assayed for the hormones of interest. Analysis included measurement of glucose, insulin, HbA1c, total cholesterol (TC), high-density (HDL-C) and low-density lipoprotein (LDL-C) cholesterol, triglycerides (TGL), AST, ALT, total estradiol (E2), total testosterone, FSH, LH, prolactin (PRL), leptin, adiponectin and oxytocin levels. Obese patients underwent an oral glucose tolerance test (OGTT) for the determination of glucose and insulin levels, with the diagnosis of T2DM determined according to the latest ADA guidelines *(ADA 2016)*. HbA1c was determined by a turbidimetric immunologic inhibition assay (TINIA; Boehringer-Mannheim, Germany); HbA1c values of 6.5% were also considered diagnostic for T2DM. Insulin resistance was calculated by the homeostatic model of insulin resistance (HOMA-IR) index as insulin

(mU/L) x [glucose (mmol/L)/22.5]. A two-site, solid-phase chemiluminescent immunometric assay or competitive immunoassay was used for insulin, E2, total testosterone, FSH, LH and PRL levels (Immulite 2000 Analyzer; DPC, Los Angeles, CA). Blood glucose and the lipid profile were measured by enzymatic assays (Roche Diagnostics GmbH, Mannheim, Germany). Serum adiponectin levels (μg/mL) were determined by an enzyme-linked immunoabsorbent assay (DRG Instruments GmbH, Marburg, Germany), the detection limit was 1.56–100 ng/mL, having sensitivity of 0.2 ng/mL and inter- and intra-assay coefficients of variations (CVs) of 2.4-8.4 and 0.9-7.4%, respectively. Serum leptin concentrations were quantified using a commercially available ELISA kit (Mediagnost GmbH, Reutlingen, Germany) with sensitivity of 0.2 ng/mL and overall inter- and intra-assay CVs of 6.8-8.3 % and 5.5-6.9% respectively. Plasma oxytocin EIA (Phoenix Pharmaceutical Europe GmbH, Karlsruhe, Germany) was performed according to the manufacturer's instructions. Plasma specimens were extracted to eliminate interfering molecules and concentrate the sample before analysis. Sample extraction was performed using a solid-phase extraction recommended by EIA kit assay manufacturers and as described by Szeto et al *(Szeto 2011)*. The lowest calibrator for this EIA kit was 0.01 ng/mL and the minimum detectable concentration was 0.06 ng/mL. The inter- and intra-assay CVs were 5.2-14.5 % and 8.7-9.1% respectively.

Statistical analysis

Statistical analyses were performed using the SPSS version 18.0 software. All data are summarized as mean and standard deviations. Continuous variables with skewed distribution were logarithmically transformed for statistical analyses. Inter-group and intra-group comparisons were performed by ANOVA. Pearson's correlation coefficients were calculated to evaluate the associations between serum oxytocin and clinical and laboratory measurements. Partial correlation was used to determine the associations after adjusting for the effects of BMI, menopause and age. The effects of covariates and covariate interactions with oxytocin were studied by the general linear model and by stepwise multiple linear regressions. Statistical tests were 2-tailed with $p<0.05$ as the threshold level of significance.

Results

Data summaries for anthropometric and biochemical variables are reported in Table 1 and 2 (pag. 72, 73). Compared to NW, OB women reported earlier menarche and more pregnancies. They also harbored lower FSH and LH levels, and higher estradiol and testosterone concentrations. Within the OB group, pre- and postmenopausal women differed for age at menarche, parity and total number of pregnancies. Compared to their postmenopausal counterpart, premenopausal women from each group showed greater values of FFM values, while %FM values were seemingly overlapping across stages. Adiposity-related measures, lipid profile and parameters of glucose homeostasis were expectedly different between OB and NW groups and 25% of OB women (7 pre- and 7 postmenopausal) were diabetic. Higher adiponectin levels were seen in postmenopausal women within each group, with significance only observed in the OB group.

Analysis of circulating oxytocin documented a significant blunting effect of menopause and high bodyweight. In the OB group as a whole, oxytocin levels were 3.5-fold lower than NW women, whereas stage-dependent differences accounted for 2.7-fold difference before menopausal and 5.8-fold difference after menopausal between NW and OB group (Figure 1, pag. 75). In OB group, oxytocin levels were below 0.1 ng/ml in 10 cases, all being postmenopausal except for one, while values were below the lowest detection limit in 3 OB cases (1 in premenopausal and 2 in postmenopausal group, respectively). In single group analyses, for both NW and OB groups, oxytocin levels were significantly lower in postmenopausal than premenopausal stage, with differences being more striking in obese conditions. As one of the postmenopausal woman from the NW group showed unexpectedly elevated oxytocin levels, the results were re-analyzed after omitting this subject from the dataset, however the differences remained unchanged (data not shown).

Bivariate analysis on the two groups as a whole showed associations between oxytocin and age at menarche, as well as menopausal status (Table 3, pag. 74), while age at menopause, menopause duration or menopause-related hormone levels showed no influence (data not shown). In our study, the association between oxytocin and age demonstrated a borderline significant ($p=0.07$). Strong inverse associations were seen between oxytocin and adiposity indices (Figure 2), i.e. leptin levels, bodyweight, BMI,

FFM, %FM and %FM Trunk, whereas associations relating oxytocin to measures of glucose homeostasis, lipids and adiponectin were feeble (Figure 2, pag. 76). Corroborating the dominant role of menopause and obesity on circulating oxytocin in our dataset, the aforementioned correlations disappeared in partial correlation analyses after controlling for such variables, either as single or aggregate covariates. Inclusion of age and/or estradiol levels as covariates did not influence the observed results. Separate correlation analyses in NW and OB subgroups showed correlations between oxytocin and age (NW: $r=-0.32$, $p<0.05$; OB: $r=-0.33$, $p<0.05$) and menopause (NW: $r=-0.28$, $p<0.05$; OB: $r=-0.37$, $p<0.05$). In covariate analysis, such correlations were independent of BMI. An association between oxytocin and estradiol levels ($r=0.28$, $p<0.05$) was only recorded in OB women.

By multivariate regression analyses, the only variables entering the regression equation for oxytocin were BMI ($\beta=-0.51$, $p<0.0001$) and menopause ($\beta=-0.28$, $p<0.001$), with a cumulative adjusted R2 value of 0.32. Confirming their inter-independent effects, the P for interaction between the effect of obesity and menopause on oxytocin was not significant ($F=0.34$, $p=0.5$). The same result was obtained when BMI, leptin levels, fat mass or estradiol levels were introduced as alternative covariates.

Discussion

Oxytocin is a labor-related hypothalamic peptide involved in social bonding, and recently has been characterized for its involvement in the regulation of energy homeostasis in animals and humans. In the present study, we documented that menopause and obesity can independently influence circulating oxytocin levels. Regardless of obesity, oxytocin levels were lower in post- than premenopausal women, while obesity was per se associated with reduced oxytocin levels, and further amplified the negative effect elicited by menopause. In our scenario, the effect of menopause and obesity appeared to be inter-independent. Together, these findings may contribute in shedding light on the mechanisms involved in weight modifications at menopause.

Mid-life period is critical for many women, with transition into menopause posing a higher risk for changes in bodyweight, visceral fat accumulation and cardio-metabolic health *(WHO 2015, Karvonen-Gutierrez 2016, Palmer 2015)*. At this stage, the physiology underlying weight accrual is important in efforts focused on the prevention of subsequent

metabolic disorders. Oxytocin governs mammary and uterine functions at labor *(Arrowsmith 2014)* and is emerging for its regulatory effects on social behavior, skeletal health, body composition and feeding habits in other stages of life *(MacDonald 2010)*. Previous studies have shown that circulating oxytocin decreases with increasing age *(Elabd 2014)* and is reportedly unchanged between genders *(Qian 2014)*. Rodent studies have shown that ovarian hormone deprivation is critical for changes in oxytocin activity and its expression in the PVN *(De Melo 2016)*, while gain in bodyweight and intra-abdominal fat accumulation in ovariectomized mice was hampered by oxytocin administration *(Beranger 2014)*. Circulating oxytocin levels, and its hypothalamic expression, was also found to be associated with fasting-feeding cycles *(Deblon 2011)*, whereas loss of expression of oxytocin or its receptors promoted obesity *(Camerino 2009, Takayanagi 2008)*. Finally, low hypothalamic expression of oxytocin was found in animal models of genetic obesity, such as single-minded 1 (Sim1) haplo-insufficiency or MAGED1 deficient mice *(Holder 2000, Dombret 2012)*, and their human homologs *(Swaab 1995)*.

Our investigation provides novel evidence that menopause is associated with lower oxytocin levels regardless of obesity, and that this relationship is amplified by obesity. The magnitude in the difference between oxytocin levels in obese and lean women recorded after menopause nearly doubled that seen prior to menopause. While no hormonal, metabolic or anthropometric variable emerged as unconditional mediators of menopause effect on oxytocin, this latter was associated with estradiol levels in the obese group, possibly due to the enhanced aromatase activity *(Palmer 2015)*. Similarly, previous reports showed that estradiol increases oxytocin levels in women *(Chiodera 1991)*, and both oxytocin and its receptors are positively regulated by estrogens in the central nervous system *(McCarthy 1995)*. We are therefore inclined to consider estradiol variations as the potential main regulator of oxytocin levels across menopause. Notably, oxytocin dynamics paralleled modifications of fat-free mass across the menopause more tightly than other body or metabolic measures. While the significance of this finding remains unclear, it is worth mentioning that oxytocin plays a role in muscle tissue regeneration and homeostasis, while inhibition of oxytocin signaling or genetic lack of oxytocin alters muscle regeneration and causes premature sarcopenia *(Elabd 2014)*. Further studies are required to clarify this issue.

One key finding of our study resides in the evidence that oxytocin concentrations were significantly affected by obesity and, overall, it decreased by 3.5-fold in obese compared to lean women. As mentioned earlier, this difference was particularly robust after menopause. Several indices of bodyweight were also found to strongly associate with oxytocin levels, including BMI, FM, leptin levels and, less significantly, glucose and lipid homeostasis. Our findings agree with previous results obtained in obese patients and T2DM *(Qian 2014)*, but disagree with results obtained in obese subjects undergoing gastric binding where oxytocin levels were higher than in controls *(Stock 1989)*. However, we are inclined to explain this latter divergence as the result of technical drawbacks. Our procedures of extraction, in fact, enabled us to prevent the detection of nonspecific immunoreactive molecules, thus increasing analytical accuracy *(Breuil 2014, Szeto 2011)*.
In the search for potential explanatory mechanisms related to obesity, we noted a strong inverse relationship between oxytocin and leptin levels in both groups as a whole, but not in single study groups. This relationship is currently argued *(Perello 2013, Ott 2013)*, and was shown to be positive in overweight premenopausal women with T1DM *(Kujack 2015)* and non-obese postmenopausal women with osteoporosis *(Breuil 2014)*. Although leptin and oxytocin act centrally to inhibit food intake *(Ott 2013)*, and leptin effects are partly mediated by oxytocin-expressing neurons within the PVN *(Blevins 2004)*, animal and human studies have demonstrated that obesity impairs the hypothalamic interplay between these peptides *(Morton 2012, Chiodera 2000)*. Oxytocin administration to diet-induced obese mice or obese humans was shown to bypass leptin resistance and reduce caloric intake without affecting leptin levels *(Lawson 2015, Fenselau 2017, Thienel 2016)*. These and our results prompt evidence that oxytocin signals bodyweight independent of the leptin-sensitive circuitry.

Conclusions

In summary, this is the first study to show that oxytocin undergoes the negative effect of obesity and menopause, and that this regulation is inter-independent. Further studies are warranted to clarify if menopause intervenes to regulate the energy-regulating actions of oxytocin, and thus contribute to explain oxytocin involvement in weight modifications at menopause.

Tables

Table 1. Anthropometric characteristics in normal weight women and women with obesity, after stratification in the pre-menopausal (Pre) and post-menopausal (Post) subgroups.

	Normal weight women (N=53)			Obese women (N=56)		
	Menopause status					
Parameters	**Pre (N=27)**	**Post (N=26)**	**P**	**Pre (N=28)**	**Post (N=28)**	**P**
Age (yrs)	47.6±3.6	56.7±2.7	<0.001	44.7±3.9*	55.5±3.8	<0.001
Age at menarche (yrs)	12.6±1	12.5±1.6	ns	11.9±1.8	11.1±0.4*	<0.05
Number of pregnancies	1.33±0.9	1.27±0.87	ns	1.25±1.2	2.25±1.4*	<0.05
Weight (Kg)	62.52±9.6	57.5±6.4	0.03	116.8±9.6§	113.2±13.6§	ns
Height (cm)	159.3±6.7	158.1±8.0	ns	158.8±7.2	157±6.2	ns
BMI (kg /m2)	23.4±3.2	21.9±2.8	ns	46±4.2§	46.1±4.7§	ns
FFM (kg)	38.5±5.2	35.6±3.4	<0.05	53.3±5.1	49.8±6.7§	<0.05
FM (%)	37.7±6.6	36.4±8.4	ns	51.8±3.4§	53.2±3.1*	ns
FM Trunk (%)	36.1±8.6	35.3±9.9	ns	51.2±4.5*	53.5±4.3*	ns

*Results are expressed as mean ± standard deviation (SD). For statistical significance: ns, not significant; *, p<0.05 and §, p<0.001 between normal weight and obese groups.*
For abbreviations: BMI body mass index; FM fat mass; FFM fat-free mass; FM Trunk is indicated as the regional percentage of fat mass of trunk.

Table 2. Baseline hormone and metabolic characteristics in normal weight (NW) and obese (OB) study groups, after stratification in the pre-menopausal (Pre) and post-menopausal (Post) subpopulations.

	Normal weight women (N=53)			Obese women (N=56)		
	Menopause status					
Parameters	**Pre (N=27)**	**Post (N=26)**	**P**	**Pre (N=28)**	**Post (N=28)**	**P**
Oxytocin (pg/mL)	3.5±2.1	2.3±1.9	<0.05	1.31±1.5§	0.4±0.6§	0.005
Leptin (ng/mL)	21.5±11.7	16.5±10.9	ns	75.5±21.8§	78.8±29.7§	ns
Adiponectin (µg/mL)	17.4±9.0	21.8±9.7	ns	8.11±3§	11.2±5.3§	0.01
Glucose (mg/dL)	88.59±11.5	87.88±8.8	ns	102±16*	111.9±32*	ns
Insulin (mg/dL)	8.3±4.8	7.1±3.7	ns	14.8±8.4*	14.1±8.5*	ns
HOMA-IR	1.86±1.3	1.56±0.88	ns	3.8±2.4§	3.8±2.3§	ns
HbA1c (%)	5.34±0.26	5.37±0.24	ns	6.1±0.5§	6.5±1.4§	ns
TC (mg/dL)	206±37	214±29.7	ns	211±39.6	199±37.6	ns
HDL-C (mg/dL)	65±16.5	71.7±17.4	ns	41±9.3§	42.5±8.4§	ns
LDL-C (mg/dL)	130.6±34.7	131±29.4	ns	139.2±36.1	126±34.3	ns
TGL (mg/dL)	90.7±46	94±41	ns	151.5±69.8§	149.7±54*	ns
AST (U/L)	14.8±5.7	16.9±6.7	ns	28.1±14.4§	26.4±11.1*	ns
ALT (U/L)	16.7±2.9	19.2±4.3	ns	23.4±10.3§	20.8±5.4	ns
FSH (U/L)	21.48±30.5	89.5±23.9	<0.001	6.5±5.4*	45.7±22.3§	<0.001
LH (U/L)	14.1±14.9	43.8±13.1	<0.001	5.04±4.3*	25.9±10.7§	<0.001
PRL (mcg/L)	18.2±10.2	11.2±4.9	0.003	21.5±11.3	13.4±8.8	0.005
E2 (ng/L)	101.4±62.9	28.9±8.1	<0.001	110.2±80	40.2±12.3§	<0.001
Testosterone (nmol/L)	0.8±0.2	0.9±0.5	ns	1.1±0.5*	1.2±0.6	ns

*Results are expressed as mean ± standard deviation (SD). For statistical significance: ns, not significant; *p<0.05 and §p<0.001 between normal weight and obese groups.*
For abbreviations: HOMA-IR, homeostatic model of insulin resistance; TC, total cholesterol; LDL, low density lipoprotein; HDL, high density lipoprotein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; FSH, follicle stimulating hormone; LH, luteinizing hormone; PRL, prolactin; E2, total estradiol.

Table 3. Bivariate correlation analysis between oxytocin concentrations and parameters of interest in the population as a whole and sub-grouped as normal weight and obese women.

	Whole Population (N=109)		**Normal-weight women (N=53)**		**Obese women (N=56)**	
Parameters	**r**	**p**	**r**	**p**	**r**	**p**
Age (yrs)	-0.17	0.07	-0.32	0.02	-0.33	0.01
Age at menarche (yrs)	0.31	0.001	0.165	ns	0.13	ns
Menopausal status	-0.26	0.005	-0.28	0.04	-0.37	0.005
Weight (kg)	-0.48	0.001	-0.08	ns	0.17	ns
BMI (kg/m2)	-0.50	0.001	-0.09	ns	0.05	ns
FFM (kg)	-0.40	0.001	0.01	ns	0.1	ns
FM (%)	-0.49	0.001	0.01	ns	0.06	ns
FM Trunk (%)	-0.48	0.001	-0.17	ns	-0.13	ns
Glucose (mg/dL)	-0.24	0.013	0.26	ns	-0.21	ns
Insulin (mg/dL)	-0.19	0.04	0.04	ns	0.09	ns
HOMA-IR	-0.23	0.016	0.07	ns	0.04	ns
TC (mg/dL)	0.17	0.07	0.16	ns	0.19	ns
HDL-C (mg/dL)	0.41	0.001	0.08	ns	-0.01	ns
LDL-C (mg/dL)	0.12	ns	0.15	ns	0.20	ns
TGL (mg/dL)	-0.228	0.017	-0.005	ns	0.09	ns
E2 (ng/L)	0.132	ns	-0.16	ns	0.28	<0.05
Leptin (ng/mL)	-0.45	0.001	-0.13	ns	0.005	ns
Adiponectin (µg/mL)	0.22	0.02	-0.06	ns	-0.18	ns

Parameters related by significant associations are indicated in bold character. For abbreviations: BMI, body mass index; FM, fat mass; FFM fat-free mass; FM Trunk is indicated as the regional percentage of fat mass of trunk; HOMA-IR, homeostatic model of insulin resistance; TC, total cholesterol; LDL, low density lipoprotein; HDL, high density lipoprotein; TGL, triglycerides; E2, total estradiol.

Figures

Figure 1. Plasma oxytocin levels in pre-menopausal and post-menopausal normal weight (Pre-NW and Post-NW, respectively) and obese (Pre-OB and Post-OB, respectively) women. Results are expressed as mean ± SD.

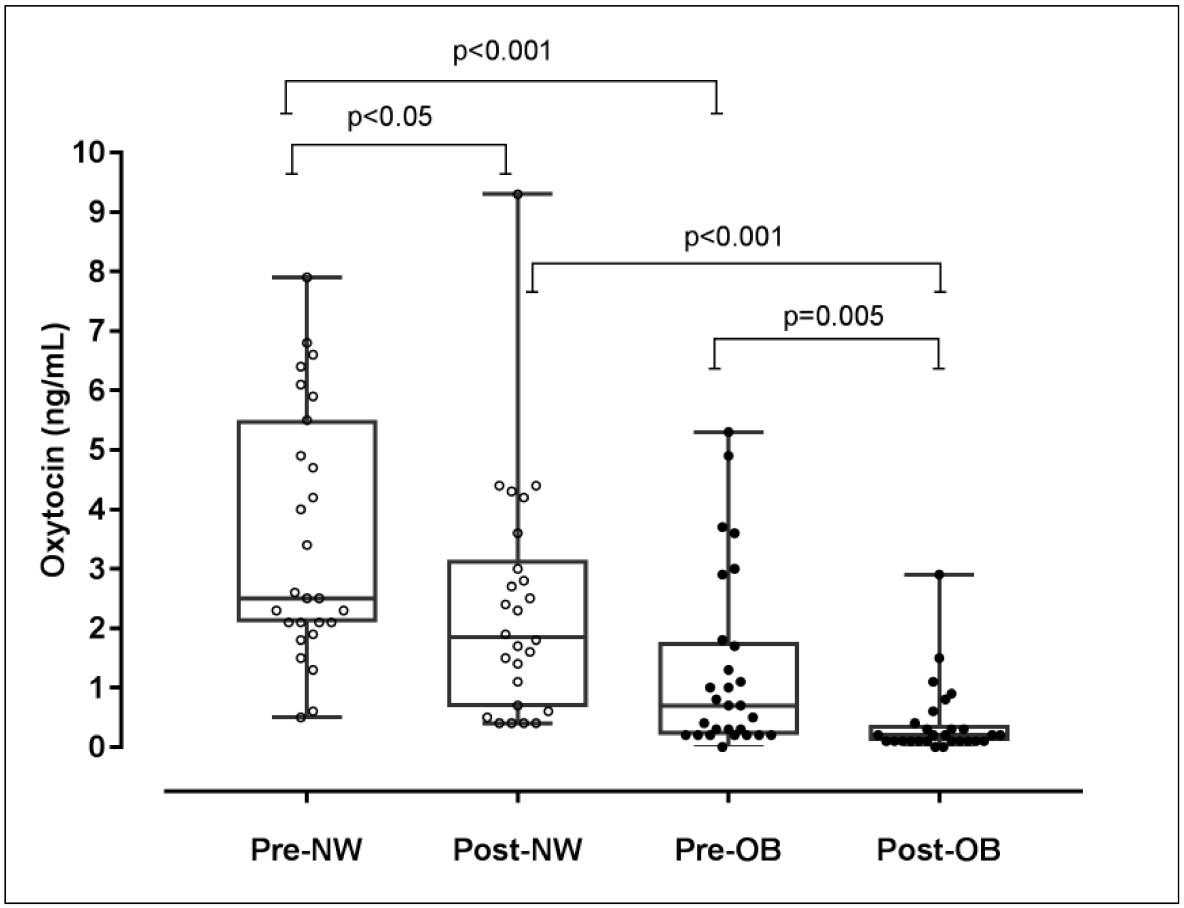
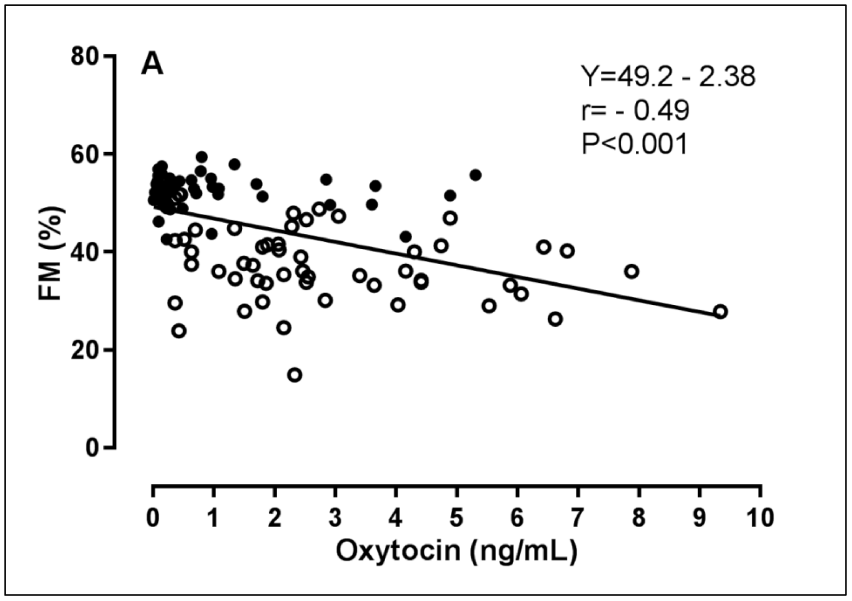


Figure 2. Relationship between oxytocin percent fat mass (FM, panel A), leptin (panel B) and adiponectin (panel C). Open circles, control group; closed circles, obese group.



Non-surgical ablative therapies for inoperable benign insulinoma

Embolization for Insulinoma in Complicated Severe Obesity: Description of an Inoperable Case and Review of the Literature

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Abstract

Purpose. Benign insulinoma is the most common functioning neuroendocrine tumor of the pancreas. The gold-standard therapeutic approach for insulinoma is surgery, which allows for tumor removal, histology and immunochemical analyses. If surgery is not feasible, minimally invasive ablative procedures performed by interventional radiology can lead to partial or complete remission of hormone hypersecretion and tumor control in insulinoma patients.

Methods. We performed a review of existing literature on non-chemotherapeutic/ radioactive ablative techniques employed for the treatment of benign, otherwise inoperable, pancreatic insulinoma. For this purpose, feasibility, effectiveness and safety of ablative treatments for pancreatic insulinoma were reviewed from literature data published from 1982 to date.

Results. A total of 44 insulinoma cases treated with non-surgical ablative techniques were desumed, and divided as follows: 7 cases of tumor embolization, 26 ethanol ablations, 7 radiofrequency ablations, 2 high intensity focused ultrasound ablation, 1 irreversible electroporation and 1 percutaneous microwave ablation. Most cases involved single insulinoma, predominantly located in the pancreas head and body. In the majority of patients, ablation was chosen instead of surgery due to severe comorbidities. After an average follow-up of 16 months, the overall success rate of non-surgical ablative treatments of insulinoma was 84%, the recurrence/persistence rate was 16%, and transient adverse events were noted in 23% of cases. Adverse events were usually self-limiting and medically manageable.

Conclusions. Non-surgical ablation is a feasible, safe and repeatable procedure in patients with pancreatic insulinoma, who are not candidate to surgery or refuse it. Partial or complete control of symptoms and tumor growth is experienced by the majority of patients.

Background

Insulinoma is the most common functioning neuroendocrine tumor of the pancreas (pNET), and accounts for 20–30% of all pNETs *(Service 1991)*. Insulinoma is usually sporadic and develops as a benign solitary intrapancreatic lesion, whereas ectopic or aggressive insulinoma is rare *(Grant 2005, Mehrabi 2014)*. The clinical hallmark of insulinoma is the Whipple's triad, which typically includes glucose-responsive hypoglycaemia with neuroglycopenia *(Pourmotabbed 2001)*. Because snacking reliefs recurrent hypoglycaemic episodes, insulinoma patients may increase calorie ingestion and increment their bodyweight or develop obesity in up to 40% of cases, thus mimicking the subtle course of the metabolic syndrome *(Dizon 1999, Goldin 2008, Inadera 1993)*.

Upon radiological identification and localization *(Morera 2016)*, surgery by enucleation or distal pancreatectomy represent the therapeutic mainstay in nearly 98% of insulinoma cases *(Mehrabi 2014)*. Nevertheless, postsurgical complications can develop in 16–43% of patients, particularly if these are abdominally obese *(Grant 2005, Arnold 2007, Solcia 1997)*. Pharmacological options, such as diazoxide, calcium channel blockers, hydrochlorothiazide and somatostatin analogues can aid disease management in patients who are inoperable, show persistent postsurgical disease, or necessitate clinical improvement to undergo surgery safely *(Tucker 2006)*. Unresponsiveness or intolerance to medical therapies usually prompt to explore alternative therapies for insulinoma.

According to the European Neuroendocrine Tumor Society guidelines *(Falconi 2016)*, the use of non-surgical ablative therapies, such as ethanol injection or radiofrequency, can be successfully attempted in patients with localized insulinoma who are not candidate to surgery. These procedures employ the endoscopic or percutaneous approach, and can be assisted by endoscopic ultrasonography (EUS) or CT guidance. To date, there is sparse evidence on the feasibility, effectiveness and safety of non-surgical ablative therapies for insulinoma *(Davì 2017)*. In this review, we sought to summarize the literature collected between 1982 and 2017 on non-surgical ablative approaches in patients with benign insulinoma. The search for relevant original publications and case reports in English was performed using PubMed, Embase and Google Scholar. The ablative procedures included trans-catheter arterial embolization (TAE), ethanol ablation (EA), radiofrequency ablation (RFA), high intensity focused ultrasound ablation (HIFU), percutaneous microwave

ablation (PMA) and irreversible electroporation (IRE). An overall summary of data published so far is reported in Table 1 (pag. 87, 88). As an index case, we illustrate the outcome of trans-catheter embolization in an elderly obese patient with multiple comorbidities.

Trans-catheter arterial embolization

Trans-catheter arterial embolization with particles coated with chemo- or radio-therapeutic agents is usefully employed in several confined malignant diseases *(Huo 2015)*. In vascular, urogenital and non-malignant endocrine conditions, TAE has been proven as a useful alternative to surgery to achieve partial or definitive cure *(Ginat 2010)*.
Between 1982 and 2014, TAE has been reportedly performed in six insulinoma-bearing patients (Table 1, pag. 87, 88) having an average age of 51 years (range 30–84 years) *(Moore 1982, Uflacker 1992, Rott 2008, Peppa 2009, Balawejder 2014)*. Reasons for TAE included refusal or failure of surgery or emergency procedure. No pre-intervention cytology was reported in any of the cases. The success rate of TAE was 67% while disease recurrence was documented in two cases, one of whom suffered from MEN-1. Post-procedure complications included abdominal pain, increase in liver enzymes, mild pancreatitis and transitory mild diabetes.
In our index case, TAE was performed in 2007 by dedicated interventional radiologists (BD and VT) on a 72-year old female patient bearing an isolated cephalo-pancreatic 1.7 × 1.5 cm insulinoma (Figure 1, pag. 90). This patient presented with incoercible obesity, unstable hypertension with hypertrophic cardiomyopathy, moderate ventilatory failure and obstructive sleep apnea, and was unresponsive to octreotide and mildly responsive to high-dose diazoxide (800 mg daily). Selective trans-catheter embolization was performed using tris-acryl gelatin-coated 40 µm microspheres on two separate steps, one year apart (Figure 1, pag. 90). The procedure was well tolerated and led to partial tumor necrosis with near-complete recovery from hypoglycemia. After 10 year follow-up, this patient has achieved a marked clinical improvement (Table 2, pag. 89), despite a mild residual disease which is well controlled by low-dose diazoxide therapy (200 mg daily). Regular CT scans performed during follow-up did not show significant dimensional

changes of the pancreatic lesion, showing a size of 1.6 × 1.3 cm at the last CT scan performed in March 2017.

Considering also this index case, the final success rate of TAE was 57%. Based on ours and others' experience, TAE represents a feasible and repeatable procedure in severely complicated patients with insulinoma, and can be associated with transient and self-limiting adverse events.

Ethanol ablation

Ethanol ablation is a minimally invasive approach successfully used to treat benign lesions, to achieve neurolysis in chronic pain, and to control growth of unresectable malignancies *(Zhang 2013)*. Recently, EA by EUS guidance has been shown to treat safely and effectively localized pancreatic tumors in patients who are poor candidates to surgery *(Zhang 2013)*.

A total of 26 insulinoma cases have been treated so far by EA (Table 1, pag. 87, 88) *(Jurgensen 2006, Deprez 2008, Vleggaar 2011, Levy 2012, Lee 2013, Qin 2014, Yang 2015, Paik 2016, Trikudanathan 2016, Burghardt 2017, Lages 2017)*. The average patients' age was 63 years, and nearly 40% of patients were aged >75 years. Reasons for EUS-guided EA included morbid obesity, clinical impairment for comorbidities, intraoperative complications, surgery refusal and elective therapy. EUS-FNA or biopsy with cytological and immunohistochemical analysis was only assessed in eight cases. Overall success rate of EA was 85% and disease recurrence was only observed in 15% of patients. Of note, successful treatment was achieved in two cases bearing multiple insulinomas (one of whom suffered from MEN-1). Literature data shows variability on the final ethanol concentration, ranging between 95 and 99%, and on the injected volume of ethanol, which should be not greater than half of the tumor volume per single interventional session *(Qin 2014)*. Post-procedure complications included localized abdominal pain, pseudocysts, and mild elevation of pancreatic enzymes in 19% of cases. However, symptoms typically subsided within 48 h following the procedure. Rare but medically manageable complications included late occurrence of hematoma and duodenal ulceration *(Deprez 2008)*. A relationship between occurrence of complications and the operator's experience has been suggested *(Qin 2014)*. Together, current literature

documents that EA of insulinoma is clinically effective, minimally invasive and at low complication risk when compared to surgical treatment *(Jurgensen 2006, Deprez 2008, Vleggaar 2011, Levy 2012, Lee 2013, Qin 2014, Yang 2015, Paik 2016, Trikudanathan 2016, Burghardt 2017, Lages 2017)*. Potential limitations or caveats to account for are constituted by small lesions, proximity to blood vessels, and risk of haemorrhage *(Qin 2014)*.

Radiofrequency ablation

Radiofrequency ablation is a well-established minimally invasive procedure that is usually performed under local anaesthesia and mild sedation, and is successfully used to ablate dysplastic and neoplastic tissue via local thermal coagulative necrosis *(Rustagi 2017)*. Despite its widespread application for the treatment of malignancies, RFA has been rarely used to treat pancreatic diseases due to an intrinsic risk of complications derived from an increased sensitivity of pancreatic tissue to thermal injury and the proximity to vascular and biliary structures *(Rustagi 2017)*.

Seven case reports have been so far published of insulinomas treated by RFA (Table 1, pag. 87, 88), 5 of which by EUS-guided RFA *(Limmer 2009, Prochàzka 2012, Waung 2016, Lakhtakia 2016, Bas-Cutrina 2017)*. Average patients' age was 60 years, and most were suffering from clinical comorbidities such as obesity, ischemic cardiomyopathy, atrial fibrillation, chronic pancreatitis or concomitant neoplasms. EUS-FNA with cytological and immunohistochemical analysis was performed in two cases. The overall success rate of RFA was 100%, and no case of disease recurrence was noted. Post-procedure complications included transitory diabetes in one case *(Prochàzka 2012)*. Although limited by the small sample size, current studies suggest that RFA is a technically feasible, safe and highly effective therapeutic methodology in selected patients with insulinoma, in whom surgery is not an option *(Limmer 2009, Prochàzka 2012, Waung 2016, Lakhtakia 2016, Bas-Cutrina 2017)*. There is growing interest on pancreatic RFA, yet a few theoretical concerns and limitations should be reckoned, such as the lack of standardization and the need for dose–effect dosimetry studies. Sorting out these issues could help to clarify the relationship between energy delivery, duration of application and

the zone of ablation produced to safely and effectively ablate pancreatic insulinoma *(Rustagi 2017)*.

High intensity focused ultrasound ablation

High intensity focused ultrasound ablation has been recently introduced for the treatment of pancreatic cancer *(Wu 2005)* and is receiving increasing interest for the treatment of several primary tumors and metastasis *(Orgera 2010)*. Wu et al. reported that HIFU is safe and feasible for the treatment of patients with pancreatic cancer, without the evidence of post-procedural complications *(Wu 2005)*. HIFU achieves ablation by means of focused US energy from an external source that is targeted within the body, resulting in thermally induced localized necrosis *(Orgera 2011)*. Orgera et al. described the use of HIFU for ablation of two insulinomas, demonstrating that this technique is a clinically successful procedure capable of improving disease control and quality of life in inoperable patients *(Orgera 2011)*. In one case, benignity was defined through biopsy with histological examination. An important limitation of this methodology is the need for general anaesthesia in most cases. Therefore, this therapeutic approach should reserved for the treatment of patients whose symptoms cannot be controlled by medical therapy and who are not candidates to surgery or any other minimally invasive treatment *(Orgera 2011)*.

Percutaneous microwave ablation

Percutaneous microwave ablation has been used as a palliative treatment modality for locally advanced pancreatic cancer *(Lygidakis 2007)*. Tissue-specific advantages of PMA have been identified in the liver, kidney, lung, and bone, while experience in the treatment of pancreatic lesions is limited. Among thermal ablative techniques, PMA shows several potential advantages over RFA when applied to pancreatic mass ablation, including faster heating over a larger volume of tissue with less susceptibility to heat sink effects and high impedance tissues, ability to use multiple applicators, and independence from grounding pads *(Chen 2015, Lubner 2010)*.

Its employment for the treatment of insulinoma has been described only in the case of a 60-year old patient, who underwent PMA because of complicated obesity *(Chen 2015)*. Benignity was established on the basis of EUS-FNA with cytological and immunohistochemical analysis. In this patient, the procedure was successful and no recurrence nor post-procedure complication was reported (Table 1, pag. 87, 88). Indeed, larger studies and extended follow-up are needed to determine the effectiveness of this treatment for insulinoma.

Irreversible electroporation

Irreversible electroporation is a novel non-thermal ablative technique mainly used for treating liver tumors and pancreatic adenocarcinomas in patients who are not eligible to surgical or conventional ablative treatments *(Dunki-Jacobs 2014)*. This technique uses high-voltage electric pulses directed to tumor cells resulting in the creation of nano-pores within the phospholipid bilayer of cell membrane, ultimately inducing apoptotic cell death *(Ierardi 2014, Scheffer 2015, Philips 2013)*. It is considered superior to other ablative methods because it preserves vital structures such as vessels, nerves or ducts *(Scheffer 2015, Philips 2013, Paiella 2015)*. IRE yields potential benefit in locoregional palliation and overall survival in the locally advanced, non-metastatic pancreatic cancer *(Moir 2014)*. Complications include bleeding, bile/pancreatic leak, portal vein thrombosis, duodenal perforation, pancreatitis and cardiac arrhythmias *(Martin 2013)*. The employment of this method for the treatment of benign insulinoma has been described only in the case of a 29-year old patient, who underwent IRE and developed mild pancreatitis after the procedure, which was treated conservatively *(Papamichail 2016)*. Benignity was established on the basis of EUS-FNA with cytological and immunohistochemical analysis. In this patient, resolution of the insulinoma-related symptoms occurred without disease recurrence. Indeed, larger studies and extended follow-up are needed to confirm the effectiveness and safety of this type of treatment for benign insulinoma.

Conclusions

Islet cells are surrounded by a rich blood supply and receive about 10–15% of the pancreatic blood flow *(Horton 2006)*, therefore, insulinoma is a suitable target for non-surgical ablative approach for the treatment of patients who are at increased anesthesiological/surgical risk, or at high risk of post-surgical complications. In terms of success and adverse events, most cases of non-surgical ablation described so far have focused on isolated and, rarely, multiple insulinomas, with diameter up to 75 mm, mostly located in the pancreas head and body. Benignity was reportedly established on the basis of endoscopic ultrasound (EUS)-fine-needle aspiration (FNA) or biopsy with cytological and immunohistochemical analysis only in 13 cases, thus that the lack of cytological verification of benignity remains an issue.

There are currently insufficient comparative data to discriminate which, among the non-surgical ablative techniques, achieves the best clinical outcome for the treatment of insulinoma. Collectively, EA and RFA seem to offer greater success in terms of disease remission. However, it should be borne in mind that some of the mentioned studies are possibly ongoing, and far more patients have been treated than those published; likely, this approach could be preferred over a Whipple's procedure when this is necessary because of the tumor location. Although the overall rate of complete disease control is 84%, a true success rate cannot be assessed because of the short duration of follow-up of most cases described to date *(Davì 2017)*. Patient's clinical conditions and tumor characteristics (e.g., location, dimension, distance from the pancreatic duct and vascularization pattern) may support the clinician in selecting the candidate patients and the most pertinent ablative procedure. Obesity per se does not seem to constitute a contraindication to non-surgical ablative procedures for insulinoma, with the potential exception of RFA due to deep layer of abdominal adipose tissue separating the source of RFA from the pancreatic tumor. In our 10-year index case experience, TAE provided a favourable rescue treatment in a severely obese patient with life-threatening complications. The overall reported recurrence/persistence rate was 16%, but the time to recurrence remains unknown in most cases, and longer follow-ups could help to better profile the true success rates. Adverse events were only noted in 23% of cases, and these were usually self-limiting and medically manageable. While surgery remains a therapeutic

mainstay in eligible patients because definitive cure is a treatment target for insulinoma *(Falconi 2016)*, we are inclined to suggest that non-surgical ablation of symptomatic well-defined insulinoma is a feasible, inimally invasive, safe and repeatable procedure in patients if they are elderly, or poor candidate to surgery, or if they refuse surgery. Clinical trials and longer follow-ups are warranted to define the role of these procedures in the treatment algorithm for insulinoma.

Tables

Table 1. Summary of the main case reports related to minimally invasive therapeutic procedures of benign insulinomas.

Author	N° of cases	Age	Gender	Tumor Size (mm)	Location of Tumor	Reason for the choice of treatment	N° of treatments	Post-procedure complications	Follow up (months)	Recurrence	Time of Recurrence (months)
Ethanol Ablation											
Jürgensen 2006	1	78	F	13	Body	Refusal of surgery Comorbidities	1	- Abdominal pain - Mild acute pancreatitis	34	No	-
Deprez 2008	1	78	F	20	Head	Comorbidities	1	- Mild pancreatitis - Ulcer of the duodenal wall	24	No	-
Vleggaar 2011	1	82	F	9.5	Body	Comorbidities	1	None	6	No	-
Levy 2012	8	67	M	11	Head	Refusal of surgery	1	None	16	No	-
		72	F	17	Head	Refusal of surgery	3	None	13	Yes	13
		60	M	14	Head	Refusal of surgery	1	- Fluid collection - Pseudocyst	35	No	-
		82	M	23	Tail	Comorbidities	2	None	13	Yes	13
		80	F	21	Body	Comorbidities	2	None	12	No	-
		57	F	9	Head	Refusal of surgery	2	None	38	Yes	38
		34	M	16	Head	Previous abdominal surgery	2	None	5	No	-
		79	F	15	Head	Intraoperative surgery complications	1	- Pancreatitis - Pseudocyst	7	No	-
Lee 2013	1	26	F	17 (multiple lesions)	Head, body, tail	Elective	1	None	12	No	-
Qin 2014	4	48	F	10	Head	Refusal of surgery	1	None	6	No	-
		56	F	5.4	Head	Refusal of surgery	1	None	4	No	-
		56	M	11.8	Junction of head and body	Refusal of surgery	1	None	5	No	-
		66	F	10	Body	Refusal of surgery	1	None	2	No	-
Yang 2015	4	59 (mean age)	N/A	N/A	N/A	Comorbidities Surgical failure	2 (n=1) 1 (n=3)	N/A	17.3 (mean) (range, 2-30)	1	N/A

Paik 2016	3	99	M	9	Head	Age	1	None	Not specified	No	-
		20	M	11	Head	Not specified	1	None	Not specified	No	-
		32	M	14	Head	Surgical failure	2	Abdominal pain	6	No	-
Trikudanathan 2016	1	66	M	14	Head	Comorbidities	1	None	1.5	No	-
Burghardt 2017	1	65	F	13	Junction of head and body	Comorbidities	1	None	12.5	No	-
Radiofrequency Ablation											
Limmer 2009	1	80	F	15	Tail	Surgical risk	1	None	7	No	-
Procházka 2012	1	75	F	17	Body	Comorbidities	1	Transitory diabetes	3	No	-
Waung 2016	1	70	F	18	Uncinate	Comorbidities	3	None	10	No	-
Lakhtakia 2016	3	42	M	14	Body	Comorbidities	1	None	12	No	-
		41	M	17	Genu	Comorbidities	1	None	12	No	-
		52	M	22 (multiple lesions)	Head, body, tail	Surgical risk	1	None	12	No	-
Bas-Cutrina 2017	1	63	F	10	Body	Comorbidities	1	None	10	No	-
Arterial Embolization											
Moore 1982	1	38	F	20	Head	Surgical failure	1	- Low-back pain - Nausea - ↑ in liver enzyme values	11	No	-
Uflacker 1992	2	56	M	18	Head	Refusal of surgery	2	- Abdominal pain - Hyperamylasaemia	18	No	-
		49	M	20	Body	Emergency	1	None	40	No	-
Rott 2008	1	84	F	14	Head	Refusal of surgery	1	- Abdominal pain - Mild pancreatitis - Transitory mild diabetes	12	No	-
Peppa 2009	1	30	M	30	Peripancreatic	Post-surgical recurrence	1	Abdominal pain	12	Yes	12
Our case	1	72	F	17	Head	Comorbidities	2	- Abdominal pain - Mild pancreatitis	108	Yes	48
Percutaneous Microwave Ablation											
Chen 2015	1	60	M	32	Neck	Comorbidities and limited life expectancy	1	None	3	No	-

MEN 1, Multiple Endocrine Neoplasia type 1; OSA, Obstructive Sleep Apnea; COPD, Chronic Obstructive Pulmonary Disease

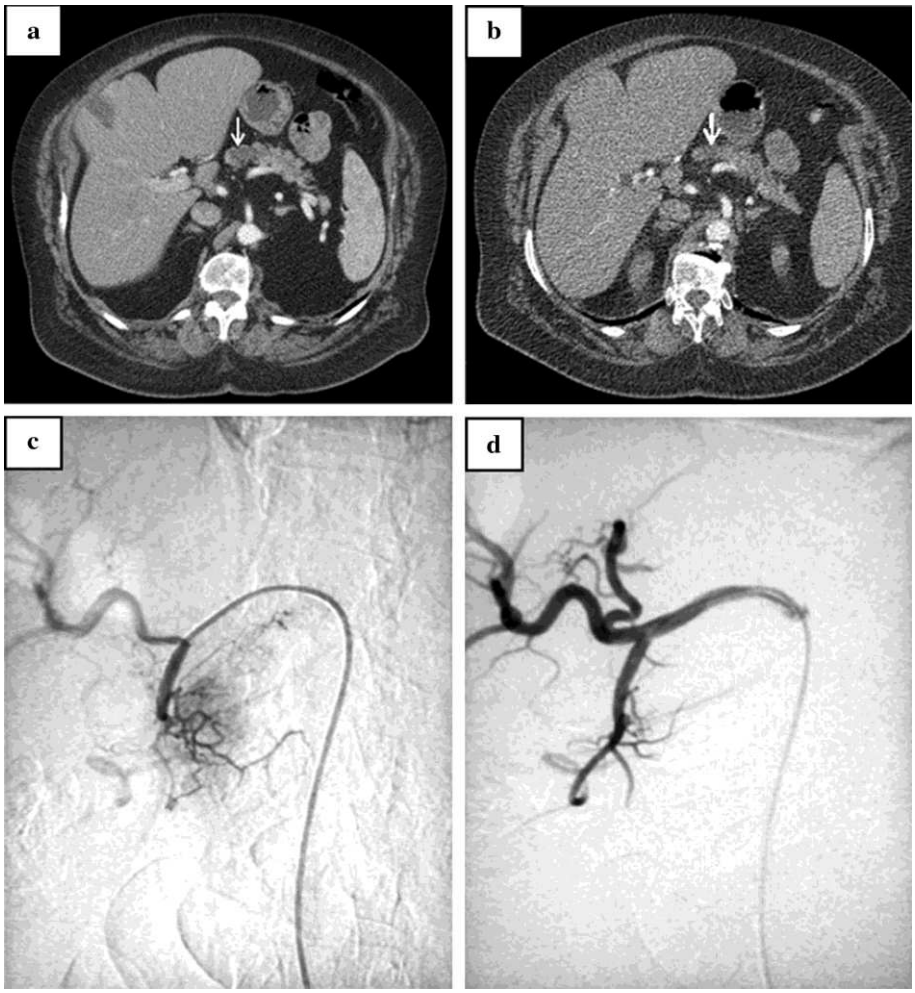
Table 2. Summary of anthropometric and biochemical data relating to pre-treatment and post-treatment period and during the last follow up evaluation.

Parameters	Before embolization (2007)	After embolization (2009)	Last follow up (2017)
Weight (kg)	119.4	82.5	104
Height (cm)	154	154	154
BMI (kg/m2)	50.3	34.8	43.8
Waist (cm)	138	116	123
Hip (cm)	125	120	123
WHR	1.10	0.96	1.00
FM (%)	52.3	47.4	48.2
FFM (Kg)	57.1	46.5	53.9
REE (kcal/24 hour)	1575	1428	na
Fasting Glucose (mg/dL)	49	67	78
Fasting Insulin (mU/L)	40.5	6.7	18.1

BMI body mass index, WHR waist-to-hip ratio, FM fat mass, FFM free fat mass, REE resting energy expenditure

Figures

Figure 1. Index case of pancreatic head insulinoma. Pre-treatment CT scan (a), post-treatment CT scan (markedly reduction in size with necrotic behaviour can be observed) (b); late arterial phase of gastroduodenal artery angiogram before embolization (c) and complete devascularisation 5 min after embolization (d).



TYPE 2 DIABETES: FROM MOLECULES FOR ITS TREATMENT TO MEDICATIONS PREDISPOSING TO ITS ONSET

Introduction

The rise in type 2 diabetes mellitus (T2DM) parallels the global increase in the prevalence of obesity. Ninety percent of patients with T2DM are overweight or obese, and over 60% of cases of type 2 diabetes can be attributed directly to excess body weight *(Hu 2001)*. Outcomes are significantly worse in type 2 diabetic patients who are also obese, including an increased risk of cardiovascular disease and all-cause mortality of 44% and 71%, respectively *(Eeg-Olofsson 2009)*. The etiology of type 2 diabetes is complex, and is the product of a combination of genetic predisposition and often, excess body weight *(Pedersen 2013)*. Excess adipose tissue increases the risk of T2DM by increasing resistance to insulin-mediated peripheral glucose uptake. The mechanisms by which this occurs are complex, involving a host of adipokines and inflammatory mediators.

Developing peptides to treat obesity and/or diabetes is a relatively recent advance, but of importance, the clinical success of this concept has been evidenced by the usefulness of intestinal peptide glucagon-like peptide-1 (GLP-1) in controlling obesity as well as diabetes *(Briones 2006)*. The human GLP-1 analogues, besides improving glico-metabolic homeostasis and promoting weight loss, are able to explicate extraglycemic effects on renal function.

In the same time, some therapies used in the treatment of conditions associated with diabetes, could increase the risk of development of diabetes.

This section aims to explore:

- The effects of liraglutide on renal function in type 2 diabetes.
- Glucose abnormalities and new-onset diabetes (NOD) in statins users.

One-year treatment with liraglutide improved renal function in patients with type 2 diabetes: a pilot prospective study

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Abstract

Background. Unlike GLP-1, liraglutide is not cleared by the glomerulus and its pharmacokinetic is not altered in patients with mild renal impairment. The aim of our study was to analyze the effects of liraglutide on renal function in patients with type 2 diabetes.

Methods. A twelve-month longitudinal prospective post-marketing study was performed. According to eGFR (estimated glomerular filtration rate) calculated with CKD-EPI equation, 84 consecutive patients were divided in Group A (eGFR>90 ml/min) and Group B (eGFR<90 ml/min). BMI, glucose, HbA1c, serum creatinine, microalbuminuria, and eGFR were evaluated at baseline and after 12 months of treatment.

Results. A reduction in fasting plasma glucose ($p<0.01$), HbA1c ($p<0.003$), BMI ($p<0.01$), and systolic ($p<0.01$) and diastolic blood pressure ($p<0.006$) was recorded irrespective of eGFR category. Concerning renal function, creatinine levels had a trend to decrease in both groups. eGFR did not change in Group A, while it increased in Group B ($p<0.05$) independently from the concomitant changes of other parameters. Moreover, seven out of 41 patients of Group B had increased eGFR levels which reached the normal values (>90 ml/min). At baseline, five patients had pathological microalbuminuria, but at 12 months three of them returned to normal albuminuria ($p<0.006$). Total microalbuminuria levels improved in both groups ($p<0.02$).

Conclusion. According to preliminary data in animals, our study shows that liraglutide is effective in preserving eGFR in diabetic patients, increasing it in those with reduced renal function. This was associated with a decrease of frequency of patients positive to microalbuminuria. Further studies are needed to confirm these data.

Background

Liraglutide is a human GLP-1 long-acting analog approved for once daily treatment for patients with type 2 diabetes.

Liraglutide, which shares 97% structural homology with human GLP-1, has a longer half-life than the native hormone and similar pharmacokinetics, with no single organ responsible for its major removal *(Malm-Erjefalt 2010)*. After a single dose of liraglutide, no intact molecules of the GLP-1 analog were found in urine or feces, suggesting slow degradation into small peptides, aminoacids, and fatty acid fragments eliminated through the liver or the kidney. Pharmacokinetic studies failed to identify a specific excretory organ, suggesting that liraglutide could be metabolized in a way similar to that of large proteins *(Malm-Erjefalt 2010)*.

Unlike GLP-1, liraglutide is not cleared by the glomerulus and its pharmacokinetic is not altered in patients with mild renal impairment *(Davidson 2011)*. Those properties may offer advantages in using liraglutide in patients with impaired renal function. In fact, dose adjustment is not required in these patients, while the use of liraglutide in patients with moderate or greater renal dysfunction is not recommended because of lack of clinical experience *(Russell-Jones 2010)*.

Several studies demonstrated that liraglutide effectively lowers glycosylated hemoglobin (HbA1c) as monotherapy or in combination with oral anti-diabetic drugs *(Madsbad 2009)*. A metaanalysis showed that mild renal impairment did not significantly affect the reduction of HbA1c or the percentage of patients reaching glycemic targets. Moreover, Liraglutide was not associated with acute changes in renal function as measured by serum creatinine supporting data on its safety in patients with mild renal impairment *(Madsbad 2009)*.

However, there is a lack of data about the renal effects of liraglutide, especially in the daily clinical practice, despite evidence supporting a role of GLP-1 in modulation of renal function *(Gutzwiller 2004)*. Different studies showed that GLP-1 is able to induce diuresis and natriuresis, but the mechanisms that regulate those effects are still unknown. GLP-1 receptor has been recently detected in porcine proximal tubular cells, where it reduces sodium reabsorption *(Schlatter 2007)*. Moreover, the infusion of GLP-1 activates an intracellular pathway mediated by cAMP/PKA which causes an increase in glomerular

filtration rate (GFR), renal plasma flow, and bicarbonate and fractional potassium excretion *(Crajoinas 2011)* in addition to its diuretic and natriuretic effects. The evidence that this peptide increases both GFR and renal plasma flow suggests that GLP-1 might exert a direct effect on renal vasculature, most likely by decreasing the resistance of the pre-glomerular capillaries *(Moreno 2002, Nystrom 2005)*.

Based on the above, the aim of our study was to analyze the effects of 12-month therapy with liraglutide on renal function in a group of patients with type 2 diabetes.

Methods

We performed a longitudinal, prospective, observational study. Study quality was assessed using the checklist "STROBE" (for 'STrengthening the Reporting of OBservational studies in Epidemiology'; additional file 1) (Supplemental Table 1). We consecutively recruited patients with type 2 diabetes, referred to the Diabetes and Endocrine Unit of the "Maggiore della Carità" hospital in Novara from August 2010 to April 2013, and all patients were evaluated at baseline and after 12 months of treatment.

We considered all eligible patients with type 2 diabetes not on target with common oral anti-diabetic drugs (HbA1c>7 % or >53 mmol/mol), to which we added a daily injection of liraglutide. Patients were excluded from the study whether they (1) had poor compliance to treatment with liraglutide (fear of daily subcutaneous injection, elderly people unable to self-administer the drug, people suffering from senile dementia and/or neurodegenerative diseases); (2) had renal failure requiring dialysis; (3) had a history of a renal disease different fromthat due to diabetes or hypertension (kidney stones, metabolic diseases, glomerulonephritis, etc.); and (4) did not gave theirconsent to undergo a regular and periodic follow-up, as required by the Italian Agency of Drugs (AIFA).

We evaluated at baseline and after 12 months of therapy BMI, glucose, HbA1c, serum creatinine, microalbuminuria, and estimated glomerular filtrate rate (eGFR), calculated with the MDRD *(Florkowski 2011)* and CKD-EPI equation *(Levey 2009)*. All the formulas are reported in Supplemental Table 2.

Patients were subdivided in Group A and Group B according to eGFR estimated with CKD-EPI, because MDRD equation has a low accuracy for an eGFR>90 ml/min/1.73 m^2

(Florkowski 2011, Levey 2009). Patients were included in Group A if they had a normal renal function (eGFR>90 ml/min) and in Group B if it was impaired (eGFR<90 ml/min). However, nine patients were discordant for eGFR if evaluated with MDRD equation, eight in group A and 1 in group B, respectively.

Height was measured by the Harpenden stadiometer to the nearest mm with the subject head in Frankfurt plane and weight using electronic scale both taken in triplicate. Averaged BMI was calculated as body weight divided by squared height (kg/m^2).

Plasma glucose levels (mg/dl; 1 mg/dl: 0,05551 mmol/l) were measured by the glucooxidase colorimetric method (GLUCOFIX, by Menarini Diagnostici, Florence, Italy). HbA1c levels were measured by the 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 with the enzimatic method of creatinine deamidase/GLDH (Adivia Chemistry-Bayer).

Statistical analysis

All data are expressed as mean ± standard deviation (SD), absolute or delta values. A sample of 60 individuals has been estimated to be sufficient to demonstrate a difference of 10.0 ml/min in eGFR with an SD of 16 with 90% power, a significance level of 95% using the Student t test, and a drop out of 10%. Distributions of continuous variables were examined for skewness and were logarithmically transformed as appropriate. Analysis of covariance was used to determine differences in subjects with and without renal impairment. Analysis of repeated measures was used to determine differences in subjects before and after the treatment with liraglutide. Covariates were sex, age, BMI, disease duration, number of drugs for hypertension, use of ACE-inhibitors, angiotensin receptor blockers (ARBs), or diuretics (yes/no). A stepwise regression was used to determine the association of changes (expressed as delta values) among each variable and delta of the others. The v2 test was used to determine difference in distribution. Statistical significance was assumed at $p<0.05$. The statistical analysis was performed with SPSS for Windows V.17.0 (SPSS Inc., Chicago, IL, USA).

Results

A population of 243 patients was firstly selected, but 159 subjects failed to respect the inclusion criteria and were excluded. In particular, (a) 58 subjects did not gave the informed consent; (b) 41 did not performed the evaluation of creatinine at baseline or after 12 months of therapy; (c) 37 showed no compliance to therapy; (d) 20 had discrepant data between our database and that of AIFA; and (e) three drop out for gastrointestinal adverse events. Of the 84 patients (age 59.9±11.1 years) who met inclusion criteria and completed the 12-month follow-up and were definitively studied, 73 of them were on therapy with 1.2 mg/day of liraglutide, ten with 1.8 mg/day, and only one with 0.6 mg/day. Liraglutide treatment was associated with metformin (42 patients), sulphonylurea (two patients), metformin plus sulphonylurea (37 patients), or repaglinide (three patients). Sixty-six patients had hypertension, of which 18 took ACE-inhibitors, 30 ARBs, and 22 diuretics (19 of them in co-administration with ACE-inhibitors or ARBs).

Complete clinical characteristics of Group A and B are listed in Table 1 (pag. 103). Group A and B were composed by 43 and 41 patients, respectively. The liraglutide dose was 1.2 ± 0.1 and 1.2 ± 0.2 mg/day in Group A and B, respectively. In Group B, 30 patients had a mild renal impairment (eGFR 60–89 ml/min), meanwhile 11 of them had a moderate renal impairment (eGFR 31–59 ml/min). In the whole population, 12-month therapy with Liraglutide induced a reduction in fasting plasma glucose (Δ -2.39±4.08 mmol/l; $p<0.01$), HbA1c (Δ -1.05±1.71%; Δ -11.20±17.74 mmol/mol; $p=0.003$), BMI (Δ -2.13±6.39 kg/m^2; $p<0.01$), and systolic (Δ -13.51±27.20 mmHg; $p<0.01$) and diastolic blood pressure (Δ -5.42±13.91 mmHg; $p=0.006$). Similar results were recorded for both GroupA and GroupB (Table 1). The reduction of systolic and diastolic blood pressure remained significant also when weighted for changes inBMI, glucose levels, and HbA1c in the whole population and in both subgroups. The number of hypertensive patients did not change (31 and 35 patients in Group A and B, respectively); however, five out of 66 patients (all of Group B) reduced the number of drugs for the treatment of hypertension. The liraglutide dose was 1.2 mg/day in all of them.

Concerning renal function, creatinine levels had a trend to decrease without reaching significance, without any differences between the two groups (Table 1, pag. 103). After 12 months of treatment, eGFR irrespective of the formula used did not change in Group A,

while increased in Group B ($p<0.05$) (Figure 1, pag. 104). The variation of eGFR irrespective of the formula

used was found to be independent from the concomitant changes of the other parameters (BMI, glucose, HbA1c, and blood pressure values) and by the number of the type of drugs for hypertension or renal impairment. Moreover, at the end of the study period, three patients of Group A showed a reduction in eGFR values below the normal range (<90 ml/min), while seven patients of Group B had increased eGFR levels which improved and reached the normal values (>90 ml/min). The liraglutide dose was 1.2 mg/day in all of these seven patients. All the three patients of Group A with a worsening in eGFR had basal eGFR<100 ml/min (respectively 94, 96, and 99 ml/min), a BMI>30 kg/m^2, dyslipidemia, and hypertension under specific treatment, but not albuminuria. The liraglutide dose was 1.2 mg/day in two of them and 1.8 mg/day in the last one. No complications or worsening of comorbidities occurred during the 12 months of observation in these three subjects.

At baseline, five patients had pathological microalbuminuria (three from Group A and two from Group B, respectively). At 12 months, three of them presented albuminuria in the normal range ($p=0.006$) and the other two of them (both from group A) showed an improvement in microalbuminuria levels. Total microalbuminuria levels improved in both groups (Δ -2.54 ± 10.32 mg/24 h; $p=0.02$) also when weighted for number of drugs for hypertension, or use of potential interfering drugs.

Discussion

Liraglutide is not predominantly eliminated by renal excretion. Some meta-analyses of Phase III studies showed that liraglutide is safe and effective in patients with type 2 diabetes at different stages of renal function *(Madsbad 2009, Marre 2009, Neumiller 2010, Rigato 2014)*.

Our study confirmed the efficacy of liraglutide in improving glycemic control in patients with type 2 diabetes, by reducing fasting plasma glucose and HbA1c levels, irrespective of eGFR, but more strikingly showed that liraglutide did not deteriorate serum creatinine levels and eGFR in patients with normal renal function and improved eGFR in those with a mild or moderate renal impairment.

First of all, we confirmed data of the registration studies on the efficacy of liraglutide about the reduction of fasting glucose, HbA1c, and BMI in a single-center daily clinical practice, where the compliance to treatment is frequently poor, number of visits each year is less, and patients' characteristics are more heterogeneous with respect to registration studies *(Neumiller 2010, Rigato 2014, Aimaretti 2009)*. The reduction of parameters was comparable to Phase III studies *(Davidson 2011, Aimaretti 2009, Henry 2011)* and also with the ABCD nationwide post-marketing study conducted in UK which included patients also with mild or moderate renal impairment *(Thong 2013)*. Moreover, the degree of clinical improvement is irrespective of renal function at baseline. It has to note that ABCD study referred to first 6 months of treatment, meanwhile our data confirmed the efficacy of liraglutide in renal impairment over 12 months of therapy.

We also observed a reduction in both systolic and diastolic blood pressure irrespective of the renal function. Data derived by previous studies *(Vilsboll 2007, Wang 2013, Robinson 2013, Katout 2014)* showed positive effects of liraglutide on the reduction of diastolic and, more strongly, systolic blood pressure irrespective of the combined therapy for the treatment of diabetes, as also an improvement of the cardiac function *(Okada 2014)*. The main hypothesized mechanism for the reduction of blood pressure was the weight decrease; however, we observed that this was independent of the changes in glucose, HbA1c, and BMI. It has to mention that GLP-1 has been demonstrated to have natriuretic and vasodilatatory effects due to inhibition of Na^+ reabsorption in the proximal tubule, attenuation of angiotensin II-induced phosphorylation of extracellular signal-regulated kinase-1/2 in renal cells, and NO-dependent and independent mechanism in endothelium *(Gutzwiller 2004, Crajoinas 2011, Wang 2013, Hirata 2009, Anagnostis 2011)*. The fact that the magnitude of changes in blood pressure was identified also in mild or moderate renal impairment and that five patients with this alteration reduced the number of drugs for hypertension leads to hypothesize that the treatment could have a further role in this condition where lower blood pressure levels are recommended and, frequently, difficult to achieve.

Furthermore, we did not observe any significant changes in serum creatinine levels, independently by daily dosing of liraglutide, even if a trend to decrease was observed. These data are in agreement with those pooled by meta-analysis and systematic reviews of clinical trials on patients with mild-to-severe renal impairment *(Davidson 2011,*

Jacobsen 2009, Giorda 2014). This is in line with those reported in the ABCD study where significance, maybe due to the higher sample size, was reached *(Thong 2013)*. However, a direct effect on kidney with a following improvement of nephropathy cannot be excluded, by considering that the activation of GLP-1 receptor was associated with the reversion of nephropathy in diabetic rats *(Liu 2012)*.

Accordingly, with this hypothesis and with data in animals, our observation of a reduction of percentage of patients with albuminuria is in agreement with other pilot studies with exenatide *(Zhang 2012)* or DPP-4 inhibitors *(Haluzik 2013)*. Finally, we observed stable eGFR levels in those with normal renal function, and, more strikingly, an improvement in those patients with a mild or moderate renal impairment with a tendency towards normal levels in a subgroup of them. To evaluate this aspect, we used two formulas, the MDRD and the CKD-EPI equations. This was done because the Cockcroft-Gault formula for calculation of eGFR has some limitations. The MDRD equation overtakes the limits due to body weight or height in patients with chronic kidney diseases. Although helpful, poorer performance of the MDRD formula was reported at low plasma creatinine concentrations (corresponding to higher levels of renal function) and tends to underestimate renal function in those with a normal eGFR>90 mL/min/1.73 m^2 *(Florkowski 2011)*. The CKD-EPI equation was validated to match the accuracy of the MDRD equation at GFR<60 mL/min/1.73 m^2 and to offer a greater accuracy at higher GFR, minimizing the over-diagnosis of chronic kidney diseases with the MDRD equation, being accurate as MDRD in the subgroup of patients with an eGFR<60 ml/min/1.73 m^2 and substantially more accurate in the subgroup of them with an eGFR>60 ml/min/1.73 m^2 *(Levey 2009)*. Our results on eGFR were evaluated with both equations and were unrelated to which formula was used to estimate the eGFR. Since the increase in eGFR levels in patients with reduced renal function is independent of the changes in other variables considered, this could address a direct effect of Liraglutide on the kidney. As discussed above, this hypothesis is supported by a few studies in animals, which showed that GLP-1 is responsible for a natriuretic effect, by reducing sodium reuptake in proximal tube and aldosterone levels in mice *(Hirata 2009)*. Moreover, the infusion of native GLP-1 activates an intracellular pathway mediated by cAMP/PKA which also causes an increase in eGFR, renal plasma flow, and bicarbonate and fractional potassium excretion *(Crajoinas 2011)*, suggesting a direct effect on renal vasculature, most likely by decreasing the resistance of

the pre-glomerular capillaries *(Moreno 2002, Nystrom 2005)*. This is reinforced by a recent re-evaluation of tissue GLP-1 receptor distribution which showed that in the kidney GLP-1 receptor was exclusively expressed in smooth muscle cells of arteries and arterioles *(Pyke 2014)*. Since the presence of microalbuminuria in diabetes implies either dysfunction of the glomerular filtration barrier and/or dysfunction in tubular reabsorption *(Haluzik 2013, Lazzara 2007)*, the improvement of eGFR could partially explain also the reduction in frequency and total amount of microalbuminuria we showed.

The results on creatinine and eGFR should be interpreted with caution since the study is of relatively short duration. However, the population is less homogenous of that selected for Phase III studies and better mirror conditions of unknown potential risks. However, the heterogeneity of patients included and the small number of those who had a mild deterioration in Group A and, foremost, of those who had a restoration of eGFR in Group B did not allow to phenotype these two subgroups which need further studies over the next few years in addition to the ongoing LEADER study *(Marso 2013)*. Since our study had relatively small numbers of patients with moderate renal impairment or under the maximal daily dosing of 1.8 mg of liraglutide, there is a need for other investigations to evaluate the safety in these specific conditions. Moreover, particular attention has to be paid to extend our data to all the class of drugs acting on the GLP-1 system, by considering that other derived GLP-1 drugs are mainly eliminated through the kidney, first of all exenatide. Another limitation of our study is the unavoidable loss of data from patients being lost to follow up or with incomplete data in real-life clinical practice. This has the potential to introduce bias of results for those patients attending visits more frequently. Nevertheless, the high concordance of our data with the results on glucose, BMI, and blood pressure of published meta-analysis on registration studies *(Davidson 2011, Robinson 2013)* is a reassuring finding. It should also be considered that the sample size was calculated for eGFR changes but not for microalbuminuria and that we weighted the analyses for the number or the type of potential interfering drugs, but the specific dose was not considered. Wider studies are warranted to better investigate these aspects.

Conclusions

Our study firstly showed that liraglutide is effective in preserving GFR in diabetic patients, increasing glomerular filtration rate in those with reduced renal function, independently by the changes in glucose levels or BMI. This effect was associatedwith a trend to decrease serumcreatinine levels and number of patients positive to microalbuminuria. Further real-life studies are needed to confirm these data.

Tables

Table 1. Characteristics of the population as a whole and according to subgroups.

	Population (n=84)		Group A (n=43)		Group B (n=41)	
	Baseline	**12 months**	**Baseline**	**12 months**	**Baseline**	**12 months**
Age (years)	59.9±11.1	60.7±10.9	52.9±9.2	53.8±9.1	66.3±8.4	67.3±8.5
Disease duration (years)	8.5±7.6	9.5±7.4	6.8±4.6	7.7±4.5	10.2±9.6	11.1±9.5
BMI (kg/m^2)	33.8±5.8	32.5±5.4[c]	35.4±6.7	33.7±6.1[c]	32.2±4.3	31.1±4.4[b]
SBP (mmHg)	154.6±24.3	142.8±20.0[c]	148.9±22.9[c]	139.8±20.2[c]	160.6±24.7	146.0±19.6[c]
DBP (mmHg)	88.1±12.2	83.7±11.2[c]	88.4±11.5	84.7±10.1[a]	87.8±13.1	82.6±12.2[b]
FPG (mmol/L)	10.7±3.4	8.4±2.3[c]	10.9±3.9	8.2±2.1[c]	9.8±2.7	8.6±2.5[a]
HbA1c (mmol/mol and%)	70.0±13.9 8.6±1.4	60.2±11.6[c] 7.7±1.1[c]	73.4±17.3 8.9±1.6	58.9±13.0[c] 7.5±1.2[c]	68.5±12.4 8.4±1.1	62.3±9.9[a] 7.8±0.9[a]
Serum creatinine (µmol/L)	72.2±20.2	70.4±22.0	60.7±12.3	59.8±13.2	83.6±21.1	81.8±23.8
Microalbuminuria (mg/24 h)	5.8±14.0	3.0±7.2[a]	7.4±18.0	4.1±9.5[a]	3.9±7.2	1.7±2.3[a]
eGFR (MDRD; mL/min)	87.4±27.1	91.9±30.6	107.7±20.6	110.3±26.5	66.1±13.7	72.5±21.3[a]
eGFR (CKD-EPI; mL/min)	87.5±20.8	89.4±21.6	104.3±8.5	103.8±12.4	69.9±14.1	74.4±18.7[a]

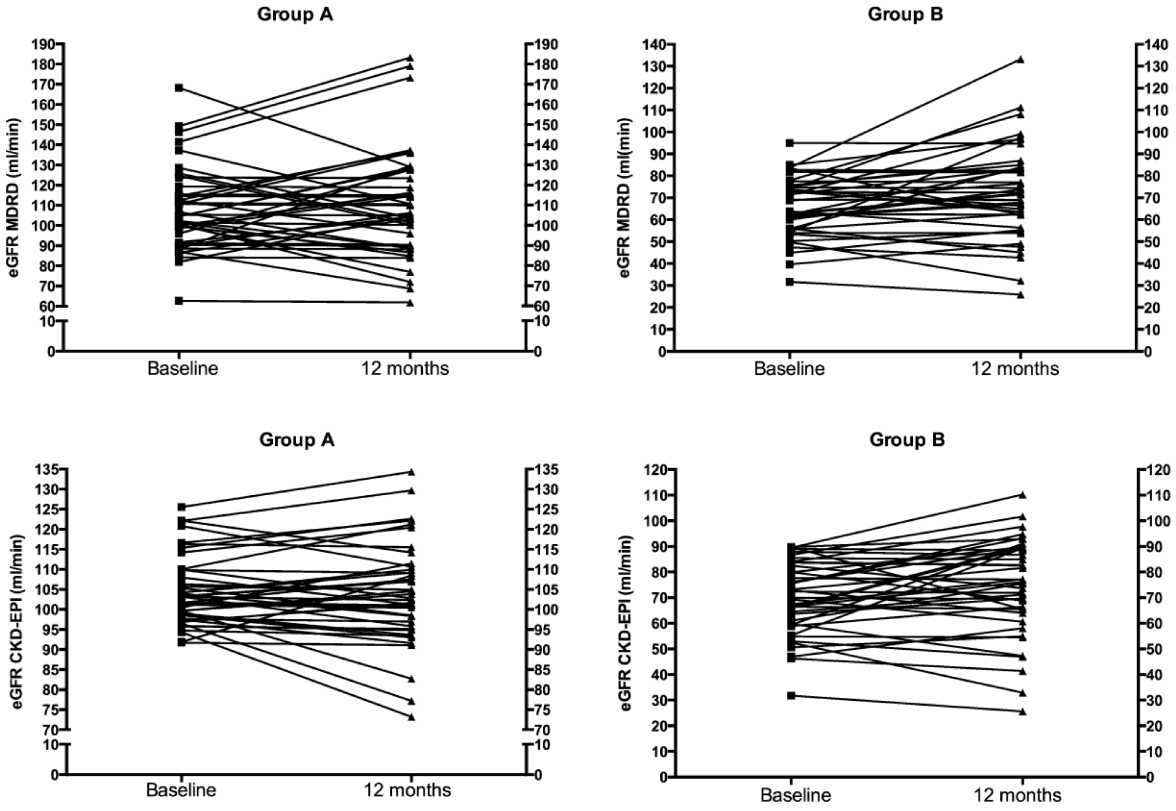
Group A: patients with a normal renal function (eGFR>90 ml/min with CKD-EPI). Group B: patients with impaired renal function (eGFR>90 ml/min with CKD-EPI). Data are expressed as mean±SD.

BMI body mass index, DBP diastolic blood pressure, eGFR estimated glomerular filtration rate, FPG fasting plasma glucose, HbA1c hemoglobin A1c, SBP systolic blood pressure.

[a]p<0.05; [b]p<0.005; [c]p<0.0005

Figures

Figure 1. Variation of glomerular filtration rate over 12 months of therapy with Liraglutide



Baseline glucose homeostasis predicts the new onset of diabetes during statin therapy: a retrospective study in the 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, the family history of diabetes (OR: 3.4, 95% CI 1.3-8.9), BMI >30 kg/m2 (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 to develop 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 develop NOD. Treatment with statin (OR: 7.5, 95% CI 1.5-37.4), in particular atorvastatin, was associated with the increased risk. In the whole population, the statin therapy (OR: 4.0, 95% CI 1.1-14.1, $p<0.020$), and in particular simvastatin and atorvastatin were 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 habits, or family history of diabetes contributed to that risk.

Background

Due to the high morbidity and mortality related to cardiovascular (CV) diseases, prevention is a priority. In Europe, around 50% of deaths are caused or are related to these pathologies (mainly coronary disease, responsible for 20% of deaths, followed by stroke *(WHO 2015)*).

Statins represent one of the most important improvements in the treatment of patients with high CV risk, as documented by many studies in both primary and secondary prevention *(CTT 2008, 2010)*. However, there is an association between statin therapy and new onset of type 2 diabetes mellitus (NOD) *(Sattar 2010, Preiss 2011, Ridker 2012, Waters 2012, Carter 2013)* which is a major CV risk factor *(Sarwar 2010)*.

In February 2012, the US Food Drug Administration (FDA) issued a warning related to statin therapy and an increased risk of NOD *(US-FDA 2016)*. However, it is useful to remember that most of the evidence arises from post-hoc analyses of studies not specifically designed to investigate NOD.

The objective 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 pre-diabetes during statin treatment on the basis of anthropometric and biochemical parameters, concomitant treatment, family history, habits, 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 *(Waters 2013, Waters 2004, La Rosa 2005, Pedersen 2005, Waters 2011)*.

Methods

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 *(STROBE 2016)*. 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 allowed. 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 should be checked and scheduled at baseline and at the last visit for family history (type 2 diabetes and dyslipidemia), habits (smoke and alcohol consumption), other diseases or treatments. They also should have evaluations of the following clinical and biochemical parameters: weight, height, waist circumference, systolic blood pressure (SBP), diastolic blood pressure (DBP), fasting glucose, HbA1c, 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 1879 subjects, of whom 1571 were excluded because failed inclusion criteria. A total of 308 subjects were included in the study. The cohort was classified according to the glucose tolerance at both baseline and follow-up. They were firstly divided into 2 groups, subjects with normal (A) and altered glucose levels (B). The subjects were further divided in patients who maintained (AA) or switched back to normal glucose levels (BA), patients who developed (AB) or 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 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 were 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 HbA1c levels ≥6.5%. Altered glucose levels 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 *(ADA 2015)*. LDL-c was determined either by 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 *(Water 2013, Waters 2004, La Rosa 2005, Pedersen 2005, Waters 2011)*, as fasting glucose >100 mg/dl (5.6 mmol/l), blood pressure levels >140/90 mmHg or treatments for hypertension, BMI >30 kg/m2, 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). HbA1c levels were measured by the 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, 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, 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 odd 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 habit 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 1879 subjects of whom 1571 patients were excluded because they did not satisfy the inclusion criteria: 1206 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. Moreover, 16 patients had been further treated with ezetimibe, 25 patients and 6 controls with PUFA-n3, 9 patients had been taken 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, HbA1c 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, pag. 117).

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 shown.

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

At the last visit of follow-up, those in group B had higher weight, BMI, waist circumference, glucose, HbA1c, blood pressure, TG, ALT, and lower T-c, LDL-c, HDL-c, and T-c to HDL-c ratio levels than those in group A (table 2, pag. 118).

By considering subjects with normal glucose levels at the follow-up (AA and BA), those treated with statins had higher BMI (24.6±3.7 vs 23.4±5.0 Kg/m^2, $p=0.050$), while 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 the 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.

Baseline clinical and metabolic characteristics of patients according to glucose levels at the 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) switched back 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 the 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), and 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/m2; 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 treatment group presented a lower prevalence of obesity (p<0.003), thiazide diuretics assumption (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), HbA1c (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.

Moreover, the AB group had lower weight (p<0.020), BMI (p<0.001), SBP (p<0.002), glucose (p<0.0001), HbA1c (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, the 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, and no alcohol assumption (OR: 0.4, 95% CI 0.2-0.9, p<0.025) decreased the risk to maintain/develop altered glucose levels or develop NOD. Thiazide assumption was marginal significant (OR: 14.1, 95% CI 0.9-210.4, p=0.052). The assumption of statins (OR: 4.0, 95% CI 1.1-14.1, p<0.029), and in particular simvastatin and atorvastatin were associated with the increased risk in the full custom models (Table 3). By using the number of TNT factors in spite of each of them, 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, and no alcohol consumption (OR: 0.3, 95% CI 0.1-0.8, p<0.010) reduced the risk to develop altered glucose levels or NOD. No effects for statins (OR: 1.1, 95% CI 0.4-3.4, p=0.753) were seen in all 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 to maintain altered glucose levels or develop NOD. Treatment with statin (OR: 7.5, 95% CI 1.5-37.4, p<0.012), and in particular atorvastatin were associated with the increased risk in the full custom models (Table 3, pag. 119).

Risk factors associated to NOD

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

In group B, to have 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 to develop NOD. Statin therapydid 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 for incident NOD after 4 years of statin treatment, particularly in those who were older *(Sattar 2010)* and on intensive-dose statin therapy *(Preiss 2011)*. 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 a slight increase in fasting plasma glucose. Moreover, the incidence of NOD in patients treated with diet was higher than other studies in European countries *(Harding 2008, Cederberg 2015)*. 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 *(Mizuno 2013)*. Cederberg et al. *(Cederberg 2015)* published similar data based on the follow-up of the METabolic Syndrome In Men

(METSIM) study conducted in men randomly selected from the Kuopio population register. They observed an increased risk of NOD in those treated with statins, but also a worsening of hyperglycemia at OGTT that represented a progressive pre-diabetic 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 result of our study is that the glucose phenotype at baseline was crucial in the risk to develop altered glucose levels with statins, whereas the risk was not present when fasting glucose levels are <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 *(Kohli 2015)*.

Our analysis of anthropometric and metabolic parameters show that patients who developed altered glucose levels or NOD had an insulin resistance phenotype at baseline. In particular, they had more central obesity, hypertension, and lower HDL-c levels and maintained these characteristics over time. These findings suggest that statins could more quickly worsen a metabolic phenotype already known to predict the risk of NOD *(Mizuno 2013, Alberti 2009, O'Neill 2015)*. Because we also observed that a higher number of TNT risk factors increased the risk, clinicians should carefully monitor patients with this phenotype because 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 proven that the risk is 3 times higher in people who have a relative affected by type 2 diabetes and 6 times higher in people who have both parents affected *(Meigs 2000, Valdez 2007)*. In our series, 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 with an alteration of glucose metabolism *(Nakanishi 2003)*. 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 *(Knott 2015)*.

Furthermore, 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 *(Mita 2007, Ray 2013, Vallejo-Vaz 2015)*. 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 *(Yu 2017)*. Moreover, the SAFEHEART cohort study showed that statins do not increase the risk of type 2 diabetes mellitus in patients with familial hypercholesterolemia *(Fuentes 2015)*. These data open the intriguing hypothesis that LDL receptor (LDLR) mutations can improve the function of pancreatic β cells *(Yu 2017)*.

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 on registries or with medical prescriptions, cox regression models for this condition were not performed. Furthermore, we studied only 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. Furthermore, many risk factors including family history of type 2 diabetes were considered.

Conclusions

In conclusion, this study confirmed that statins were 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.

Tables

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 (years)	54.0 ± 12.6	57.2 ± 11.1	0.004
Weight (Kg)	67.7 ± 12.2	75.2 ± 17.0	0.0001
BMI (Kg/m^2)	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 ± 8	111 ± 9	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)	60 ± 17	55 ± 18	0.013
LDL-c (mg/dl)	183 ± 55	168 ± 53	0.014
Non HDL-c (mg/dl)	215 ± 54	201 ± 58	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 ± 139	176 ± 105	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 ± 8	24 ± 6	0.191
ALT (U/L)	24 ± 12	28 ± 11	0.002

Abbreviations: 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/m^2)	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
HbA$_1$c (%)	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

Abbreviations: 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; WC: Waist Circumference; Group A: patients with normal glucose levels; TG: triglycerides. Group B: patients with altered glucose levels. Data are expressed as mean ± SD.

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

	All subjects			Group A			Group B		
	OR	**CI 95%**	**p**	**OR**	**CI 95%**	**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

Abbreviations. CI, confidence interval. OR, odds ratio. Group A: subjects (216) with normal glucose levels at baseline. Group B: subjects (92) with altered glucose levels at baseline. 65 patients had been treated with atorvastatin (38 A, 27 B), 85 with simvastatin (61 A, 24 B), 62 with rosuvastatin (49 A, 13 B), 37 (24 A, 13 B) with pravastatin and 16 (7 A, 9 B) with fluvastatin The models also included the following fixed factors and covariates: age, sex, BMI > 30 Kg/m2, hypertension, hypertriglyceridemia, thiazide, PUFA-n3 or ezetimibe use, 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).

CHAPTER 2

The spectrum of thyroid disorders: dysfunctions and cancer

PRIMARY HYPOTHYROIDISM:

TREATMENT AND MANAGEMENT

Introduction

Primary hypothyroidism is an insidious condition with a significant morbidity and often subtle and nonspecific symptoms and clinical signs *(Roberts 2004, Vaidya 2008)*. The earliest biochemical abnormality is an increase in serum (TSH) concentration associated with normal serum free thyroxine (T4) and triiodothyronine (T3) concentrations (subclinical hypothyroidism), followed by a decrease in serum free T4 concentration, at which stage, most patients have symptoms and benefit from treatment (overt hypothyroidism) *(Roberts 2004, Vaidya 2008, Cooper 2012).* The cause is either chronic autoimmune disease [atrophic autoimmune thyroiditis or goitrous autoimmune thyroiditis (Hashimoto's thyroiditis)] or destructive treatment for hyperthyroidism with either radioiodine or surgery which may account for up to one-third of cases of hypothyroidism in the community *(Tunbridge 1977)*. Less frequent causes include surgery and radioiodine ablation for benign nodular thyroid disease and thyroid cancer, external beam irradiation of malignant tumours of the head and neck and drugs including lithium, amiodarone and interferon *(Roberts 2004)*.

Since the 1970s levothyroxine (LT4) has become the standard of care for thyroid hormone replacement in subjects unable to produce their own thyroid hormones due to congenital, autoimmune or iatrogenic causes. LT4 has become one of the most widely used drugs worldwide, it is the most commonly prescribed drug in the United States (US),

the third most in the United Kingdom (UK), and there is evidence that its use is steadily increasing *(Rodriguez-Gutierrez 2016, Taylor 2014)*.

LT4 oral formulation has been mainly marketed as tablets whose absorption occurs in the upper intestinal tract by means of a mechanism not yet fully understood (Visser 2011). Absorption increases linearly over the first 60–90 min, and varies in the range of 60 and 80% of the administered dose *(Benvenga 1995)*. Traditionally, a dose of 1.6–1.8 mcg is considered satisfactory in order to treat hypothyroid patients *(Biondi 2014)*. However, a non-negligible rate of patients requires increased doses up to 30–40% of that estimated, whereas inappropriately high dose is cause of definite side effects *(Biondi 2014, Biondi 2008, Mercuro 2000)*.

In the last years, alternative LT4 formulations have become available in some countries and they have rapidly gained attention in clinical practice: softgel capsule and liquid solution. The softgel capsule contains LT4 dissolved in glycerin and surrounded by a layer of gelatin, while in liquid solution LT4 is dissolved in glycerol and ethanol. Pharmacokinetic studies revealed that the latter formulation might have a better in vivo bioavailability as compared to classic LT4 tablet *(Yue 2012)*. Softgel formulation has instead been proven bioequivalent in rate and exposure extent as compared to tablet formulations *(Colucci 2011)*.

This section aims to explore the management of newly diagnosed hypothyroidism in Italy as compared to the recommendations of the recent guidelines and to evaluate the patients' adherence to LT4 therapy.

An Italian Survey of Compliance with Major Guidelines for L-Thyroxine of Primary Hypothyroidism

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Abstract

Background. The adherence by endocrinologists to guideline regarding levothyroxine (LT4) therapy and the compliance of patients may impact the management of hypothyroidism. The aim of this study is to compare the adherence of Italian endocrinologists to the ATA/AACE and ETA guidelines on the management of newly diagnosed primary hypothyroidism and to validate the Italian version of Morisky-Green Medical Adherence Scale-8 items (MMAS-8) questionnaire as applied to the evaluation of the adherence of patients with hypothyroidism to LT4 treatment.

Methods. This was an observational, longitudinal, multicenter, cohort study, involving 12 Italian Units of Endocrinology.

Results. 1039 consecutive outpatients were enrolled (mean age 48 years; 855 women, 184 men). The concordance of Italian endocrinologists with AACE/ATA and ETA recommendations was comparable (77.1% and 71.7%) and increased (86.7% and 88.6%) after the recommendations on LT4 dose were excluded, considering only the remaining recommendations on diagnosis, therapy and follow-up. The MMAS-8 was filled out by 293 patients. The mean score was 6.71; with 23.9% low (score <6), 38.6% medium (6 to <8), 37.5% highly (=8) adherers; an internal validation coefficient of 0.613 was found. Highly adherent patients were not more likely to display a good control of hypothyroidism compared with either medium (69% versus 72%, p=0.878) or low (69% versus 43%, p=0.861) adherers.

Conclusions. Clinical management of hypothyroidism in Italy displayed an observance of international guidelines by Italian endocrinologists. Validation of Italian version of MMAS-8 questionnaire provides clinicians with a reliable and simple tool for the assessment of adherence of patients to LT4 treatment.

Background

Levothyroxine (LT4) oral mono-therapy is the treatment choice for primary and secondary hypothyroidism. In the last 40 years, two main developments changed the clinical practice. First, the development of the serum thyroid-stimulating hormone (TSH) radioimmunoassay discovered a high percentage of overtreatment *(Jonklaas 2014)*. First TSH assays, indeed, showed lower sensitivity compared to recent radioimmunoassay, with particular challenges in lowest limit of normal range. Second the identification of peripheral deiodinase-mediated conversion of T4 to three-iodiothyronine (T3) led to a suggestive combined association of these hormones in a sample of selected patients *(Gullo 2011)*.

The American Association of Clinical Endocrinologists (AACE), the American Thyroid Association (ATA) and the European Thyroid Association published specific guidelines dealing with primary hypothyroidism (PH) in 2013 and 2014, showing few differences, although these were of substantial magnitude *(Jonklaas 2014, Pearce 2013)*. New research suggests the need of personalized clinical management of hypothyroidism, aimed to improve diagnostic tests, therapeutic options, treatment adherence *(Hamburg 2010)* and the knowledge of underlying pathogenetic mechanisms. Three surveys evaluated the approach to PH in France and elsewhere *(Burch 2014, Delemer 2012, McDermott 2001)* but little is known about the management of PH in Italy and its concordance with clinical guidelines. Moreover, a valid, reliable and cost-effective method for evaluating the LT4 adherence is not currently available. The Morisky Medication Adherence Scale questionnaire *(Morisky 2008)*, in its more recent 8-items version (MMSA-8) shows a higher sensitivity and specificity than the previous 4-items version (MMSA-4). MMAS-8 is an easy and fast, self-reported tool, useful and reliable in the outpatient clinical setting. No previous studies validating the MMAS-8 in the context of hypothyroidism have been published.

Therefore, we performed an observational study aimed to determine the management of newly diagnosed hypothyroidism in Italy as compared to the recommendations of the ATA/AACE and the ETA guidelines. The second objective of the present study was to validate the Italian version of the MMAS-8 questionnaire for the patients' adherence to LT4 therapy.

Methods

Study design

An Italian survey regarding the diagnosis and clinical management of PH (**E**ndotrial SIE: **DI**agnosis and clinical management of **P**rimitive hypothyr**O**idism in Italy-EDIPO) was performed.

The institutional review board of the University of Modena and Reggio Emilia and the local institutional review boards of the 12 Endocrinological Italian Centers (Bari n=35, Brescia n=86, Cagliari n=46, Chieti n=48, L'Aquila n=68, Messina n=68, Mestre n=85, Modena n=112, Naples n=38, Novara n=126, Rome "Sapienza" n=227 and Rome "Sacro Cuore" n=100) approved the protocol and all subjects gave their written informed consent. This observational, longitudinal, multicenter, cohort study, led by the young Italian endocrinologists group (EnGioI) of the Italian Society of Endocrinology (SIE), used the STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) and STAndards for the Reporting of Diagnostic accuracy studies (STARD) checklists flow diagram and was carried out from September 2015 to March 2016.

The adherence to treatment was evaluated through the MMAS-8, created for the evaluation of anti-hypertensive drug adherence *(Morisky 2008)*. This is a self-reported questionnaire, which measures medication taking behaviour, starting from previous four-items scale further with additional items addressing the circumstances surrounding adherence behaviour *(Morisky 2008)*. Response categories were yes/no for each item with a dichotomous response and a 5-point Likert response for the last item. The total score of questionnaire was 8, divided in seven points for each of first seven items and one point divided in the last Likert question.

Participants

Outpatients were enrolled using the following inclusion criteria: age between 18 and 75 years and newly diagnosed PH *(Pearce 2013, Garber 2012)*. PH diagnosis was made when TSH serum levels were above the upper limit of the normal range, irrespective of fT4 and fT3 serum levels. Thus, both clinical (elevated TSH with low fT4 serum levels) and

subclinical (elevated TSH with normal fT4 serum levels) hypothyroidisms were included. Exclusion criteria were: (i) previous thyroidectomy for thyroid cancer, (ii) secondary hypothyroidism, (iii) hypothyroidism already treated with LT4 and (iv) pregnancy.

Setting and Variables

Patients were enrolled at Visit 1 (time 0). Visit 2, 3 and 4 coincided with any further follow-up visit. Socio-demographic, clinical, therapeutic management and laboratory data were collected by the clinician using electronic case report forms (eCRF) at baseline and at follow-up visits. When available, thyroid ultrasonography (US)-detectable abnormalities such as goiter and/or thyroiditis (US-thyroiditis) were recorded, according to standard criteria *(Rago 2008)*. In particular, US-thyroiditis was defined by a diffusely heterogeneous echotexture and a marked diffuse reduction of the gland echogenicity, together with admixed hypo- and hyperechoic not-nodular areas, all often associated with decreased volume of the gland.

Each endocrinologists used a therapeutic approach based on their clinical practice. The recruitment of the Centers was performed by the Coordinating center before starting the trial, considering the possibility to cover all Italian regions.

The treatment to treatment was evaluated by MMAS-8 questionnaire, which was not yet published in Italian language. For this study, the questionnaire was adapted for LT4 therapy evaluation and the Mapi company (Lyon, France) conducted the linguistic validation in Italian.

Statistical Methods

All data were collected in eCRF and a database was created at the Coordinating center. Hormonal data were converted to the same units of measurement, given the methodological differences among the reports of the laboratories. Since inclusion criteria depended on the PH diagnosis, each result was referred to the correspective laboratory reference range. Statistical analyses were performed at the Coordinating center.

The descriptive analyses were performed considering all patients enrolled in the trial, whereas the evaluation of clinical practice was performed considering both AACE/ATA

and ETA recommendations. The variables regarding LT4 treatment that were evaluated included formulations, brands, doses and dosing regimen. In addition patient who received a constant daily dose and those receiving different daily doses were considered separately in some analyses. The distribution of each variable was evaluated through the Kolmogorov-Smirnov test. The prevalence of patients in each subgroup was compared by Fisher-Yates test. Comparison of variables among different groups was performed by ANOVA univariate when they showed normal distribution, whereas Mann-Whitney or Kruskal-Wallis tests were applied for not-normally distributed parameters. Post-hoc tests were performed by Dunnet test. Parameters were compared through Spearman's correlation coefficient. For all comparisons, p values < 0.05 were considered statistically significant.

The validation of the Italian version of the MMAS-8 questionnaire was performed following different steps. First, the MMAS-8 was translated in Italian. Second, it was administered to patients included in the study and data were collected. Third, the internal validity was considered, using standard statistical procedures described by Cronbach *(Cronbach 1951)*. The Cronbach alpha is an adjusted proportion of total variance of the item scores explained by the sum of covariance between scores *(Cronbach 1951, Bland 1997)*. Thus, it ranged between 0 and 1 if all covariance elements were not negative, such as for MMAS-8 *(Cronbach 1951, Bland 1997)*. Items with higher coefficient alpha were preferred to measure target outcome *(Heo 2015)*. Internal validity was considered when the Cronbach alpha is >0.6. Fourth, the MMAS-8 reliability was considered following Cronbach's coefficient. Finally, the survey's information were revised according to the internal validity obtained. In particular, the validity of the 8-item scale was assessed through associations with TSH serum levels and according to sub-grouping of patients.

Statistical analysis was performed using the 'Statistical Package for the Social Sciences' software for Macintosh (version 21.0; SPSS Inc., Chicago, IL).

Results

Twelve Italian Units of Endocrinology were involved in the survey. 1039 patients were enrolled according to inclusion and exclusion criteria, for a total of 1430 records considering also the follow-up visits.

Study population

The baseline characteristics of the study population are reported according to gender groups in Table 1 (pag. 138). The mean age was 47.84+15.32 years, with a significant difference among Centers (p<0.001), and the majority of patients (82.3%) were females. Comorbidities were present in 161 patients (5.5%) with a higher prevalence of cardiovascular diseases (13.6% of the total) in male patients (23.4% vs 11.1%).

Hypothyroidism was incidentally discovered in 349 patients (43.1%). Other reasons for thyroid function evaluation were the presence of symptoms in 246 patients (30.4%), positive familial history in 190 (23.5%) and previous thyroid disease in 130 (16.1%). Less frequently, patients underwent thyroid function evaluation for infertility (6.4%), overweight (3.3%), tachycardia (1.8% and osteoporosis (1.7%). Overall, the 64.4% of our cohort (609 patients) had subclinical hypothyroidism.

During the follow-up, a significant decrease of serum TSH levels together with a significant increase in fT4 levels was observed (Table 1, pag. 138).

Treatment

At baseline, LT4 monotherapy was prescribed in 76.1% of patients, with a comparable distribution between sexes (76.5% in females versus 72.8% in males).The brand was specified in the 79.1% of the entire cohort according to the following reasons: consolidated personal clinical behaviour (n=509, 72.7%), patient's allergy or intolerance (n=78, 11.1%), patient's choice (n=102, 14.7%), factors interfering with the LT4 absorption (n=5, 0.7%) or difficulties in swallowing pills (n=3, 0.4%), availability of specific LT4 doses (n=3, 0.4%). LT4/LT3 combined therapy was chosen in 6 patients (0.8%), whereas a preparation containing only selenium and/or iodine integration was recommended in 114 patients (11%).

Follow-up

A follow-up visit was recommended in 876 patients (84.3%) with serum thyroid function testing in 699 patients (67.3%) and thyroid US to 368 patients (35.4%). During the entire

duration of the trial, a modification of the initial therapeutic dose was recommended in 143 patients (16.3%).

Adherence to AACE/ATA Guidelines (see table 2, pag. 139)

Diagnosis

In our cohort, serum autoimmunity against thyroid gland was evaluated in 437 patients (71.7%) with subclinical hypothyroidism, even though AACE/ATA guidelines do not recommend autoimmunity evaluation in all hypothyroid patients.

Therapy

LT4 treatment was started in almost all patients (n=237 [95.9%], 186 females – 78.5% and males 21.5%) with TSH > 10 mIU/L. The therapy was postponed in 10 patients and a re-evaluation of hormone levels was suggested.

A concordance to guidelines regarding the LT4 mono-therapy and the description of correct modality of assumption was evident.

LT4 dose

Only seven patients (5.3%, 6 females and 1 male) received the full replacement dose (1.6 µg/Kg daily), as suggested by AACE/ATA guideline, while the mean dose of LT4 suggested was 0.91 µg/kg daily. The 85.4% (n=158, 127 females and 31 males) of patients older than 50 years was treated with LT4 although the regimen provided a gradual restore of euthyroidism only in 53.4% of those patients (n=29, 22 females and 7 males). The 43.2% (n=66) of those patients without coronary heart disease and 40% (n=2) with coronary heart disease was treated as suggested by guidelines.

LT4 was prescribed in 73.9% (n=450) of patients with subclinical hypothyroidism, and in particular the recommended dose of 50-75 µg daily was suggested in 361 patients (80.2%).

Follow-up

Guideline suggests evaluation of TSH 4-8 weeks after starting or adjusting LT4 treatment, however 876 patients (84.3%, 724 females and 152 males) were evaluated after the first

visit within a mean interval of 4.29+3.36 months. Therefore, among treated patients, the guideline was strictly followed only by 146 patients (20.3%), suggesting a poor adherence to follow-up recommendations of the guidelines.

In conclusion, the adherence of Italian endocrinologists to AACE/ATA guidelines was high, on average, with a mean concordance of 77.1%. However the final percentage of concordance increased to 88.6% excluding the low adherence to dose-related items (i.e. R22.7.1: Young adult patients with overt hypothyroidism should be treated with a full replacement dose (1.6 μg/kg daily). R22.7.2: Patients >50 years with overt hypothyroidism and without coronary disease should be treated with 50 μg daily. Gradually restore euthyroidism in patients older than 50-60 years of age. R22.8: Patients with subclinical hypothyroidism should be treated with 25–75 μg, depending on the degree of TSH elevation).

Adherence to ETA Guidelines (see Table 3, pag. 140)

Therapy

Considering the entire cohort of patients with subclinical hypothyroidism, the treatment proposed and the description of the correct assumption modality was high.

LT4 dose

The starting LT4 dose was higher in younger than older subjects (p=0.002), without significant difference when patients were divided considering the degree of subclinical hypothyroidism. The mean LT4 daily dose adjusted for weight was 0.93±0.70 μg/Kg. Only 2 patients (0.5%) of those without cardiac disease were treated with the exact recommended dose of 1.5 μg/Kg daily of LT4.

On the contrary, 50 patients (12.5%) were accordingly treated, considering the suggested dose correction of 0.1 μg/Kg daily.

Patients older than 70 years were treated with a mean LT4 dose of 48.81+24.02 μg, with a high concordance with guidelines recommendations, even though only one male patient was treated with a gradual increase LT4 therapy.

Considering patients with age less than 70 years, almost all patients with TSH > 10 mIU/L (n=63, 92.6%) were treated with LT4 (mean dose of 73.70+39.08 μg). Conversely, 504 patients with TSH value < 10 mIU/L were treated in 71.4% of cases (n=360).

Follow-up

The follow-up visit was proposed in 313 treated patients, with a mean interval of 4.43±3.87 months, with a low concordance to guideline recommendations.

In conclusion, the adherence of Italian endocrinologists to ETA guidelines was 71.7% in average. However, the final percentage of concordance increased to 86.7% excluding the low adherence to dose-related items (45.5%) (i.e. recommendation 17: For patients without cardiac disease, a dose of 1.5 μg/kg daily should be use. Recommendation 18: For patients with cardiac disease or in the elderly is recommended a dose of 25 or 50 μg daily and a dose increase by 25 μg daily every 14–21 days until a full replacement dose is reached).

Patient's adherence to the treatment

MMAS-8 questionnaire was obtained from 293 patients, with a mean score of 6.71±1.42.

The internal validation of the MMAS-8, evaluated by Cronbach coefficient alpha (13) was 0.613. Moreover, the item-total correlations were greater than 0.30 for all items, suggesting that MMAS-8 could be applied to evaluation of adherence to LT4 treatment, as well (Table 4, pag. 141).

A stratification of patients according to adherence to LT4 therapy was performed and compared with thyroid function assessment. Highly adherent patients were specified based onscore of 8 on MMAS-8, medium adherers by a score of 6 to <8, and low adherers with a score of <6 (Table 5, pag. 142). TSH serum levels did not differ among high, medium and low adherent patients (p=0.139). Forty seven patients (14.7%) had at least one agent and/or condition possibly having an impact on LT4 therapy and consequently on serum TSH levels.

The MMAS-8 scores did not differ between sexes (p=0.348). The total MMAS-8 score was significantly directly related to the age (r=0.155, p=0.008). The age was significantly different among MMAS-8 categories (p=0.036), and post-hoc tests showed that low

adherent patients were significantly younger than highly adherent patients (p=0.031). The MMAS-8 score did not relate to the number of concomitant medications (p=0.098) except that highly adherent patients were more frequently treated with 3 or more concomitant medication compared to low adherent patients (p=0.016). MMAS-8 score did not differ among LT4 dosing regimens (p=0.142) and formulation (p=0.331) in the three adherence categories. Considering the time intercurrent between LT4 and breakfast, even though a post-hoc test showed a significantly longer interval in patients on LT4 tablets compared to liquid formulations (p<0.001) and soft gel (p<0.001), MMAS-8 score did not correlate with the fasting time.

Discussion

This is the first prospective, multicenter study designed to evaluate the approach to the management of PH in Italy. The study demonstrated a variable or somewhat variable approach of Italian endocrinologists towards PH, albeit in the context of a good percentage of adherence to AACE/ATA and ETA recommendation, although not complete. The second key result of the study was that it validates the Italian version of the MMAS-8 questionnaire for the adherence to LT4 treatment.

A similar adherence of the Italian endocrinologists to AACE/ATA and ETA recommendations was found (77.1% vs 71.7%). Noteworthy, these percentages must consider a low adherence to the guidelines recommendations concerning the ideal timing of follow-up visit.

Moreover, the concordance to guidelines increases to 86.7 and 88.6%, respectively, after the exclusion of the recommendations regarding the LT4 dose. This result confirms the personalized medicine approach of Italian endocrinologists, who engage to establish case by case the LT4 daily dose needed to treat PH, confirming once again the importance of the customized therapy *(Hamburg 2010)*. Indeed, the exact TSH threshold for the treatment start, the LT4 starting dose, the dosage adjustment in respect to clinical variables (i.e. age, sex, the presence of cardiovascular diseases) *(Pearce 2013, Garber 2012, Rodondi 2010)*, and the origin of hypothyroidism (iatrogenic, congenital or acquired) remain still topic of debate.

The starting LT4 daily dose depends on different factors, such as age, sex and body weight *(Roti 1993, Mandel 1993, Toft 1994, Sawin 1983)*. These variables influence each other and they could mask the real effect of the treatment *(Devdhar 2011)*, limiting the ability to design fully accepted recommendations. Both AACE/ATA and ETA guidelines do not recommend a fixed LT4 starting dose, suggesting a hypothetical starting dose, adjusted for anthropometrical features, clinical response, follow-up and laboratory determination *(Jonklaas 2014, Pearce 2013, Garber 2012)*. As in different clinical conditions, the personalization of treatment, doses and regimen seems to be the best applicable approach in PH *(Hamburg 2010)*. This approach often requires small LT4 doses and adjustment *(Brajkovich 1983)*, thus, the availability of intermediate doses of LT4 preparations could influence the clinician's decision to improve patients' adherence and endpoint achievement. The difficulty on the LT4 starting dose definition was already demonstrated worldwide *(Burch 2014)*.

Serum TSH represents the most reliable therapeutic endpoint for the treatment. Despite the discrepancies between LT4 starting dose chosen and recommended, the normalization of TSH serum levels is achieved in the vast majority of our patients at the first follow-up visit. Previously, a similar reduction in TSH serum levels was observed in a French population, despite the average LT4 dose was lower than the full-recommended replacement dose *(Delemer 2012)*. These results confirm once again the difficulty to define an exact LT4 starting dose universally acceptable. Alongside finding the exact dose, the correct time interval for follow-up visits remains a challenge in drawing and following guidelines. A relevant percentage of Italian endocrinologists do not strictly respect the recommended time interval proposed by AACE/ATA and ETA guidelines for the follow-up. This discrepancy could be explained by the difficulty in clinical practice to evaluate outpatients frequently in the setting of the Italian health care system. Our survey should be considered only a snapshot of the Italian approach to PH and the presence of biases should be carefully evaluated. For example, considering the study design and the enrolment of clinicians, and not of patients, it is not possible to completely rule out a Hawthorne effect *(Campbell 1995)* in the evaluation of Italian endocrinologists clinical habits. This effect explains the altered behaviour or performance of a clinician, resulting from awareness of being in an experiment, corresponding to the placebo effect in randomized clinical trial *(Campbell 1995, McCambridge 2014)*. Enrolled endocrinologists

were asked to fulfil the eCRF during the 6 months of survey and, although a direct control was not provided, their decisions could be influenced by the awareness of being observed.

The evaluation of LT4 treatment adherence represents the key point in the management of PH, since it seems to be the major reason for treatment inefficacy. This questionnaire is validated in several chronic treatment so far, such as osteoporosis *(Reynolds 2014)*, type 2 diabetes mellitus *(Sakthong 2009)*, and Parkinson's disease *(Fabbrini 2013)*, but not for LT4 therapy adherence. EDIPO was the first attempt to use the MMAS-8 questionnaire in the setting of PH, finding a high internal consistency of the Italian version of MMAS-8 also in this field, using the Cronbach's coefficient. This coefficient quantifies how faithfully the questionnaire items represent the outcome construct of the instrument is aiming to measure *(Cronbach 1951)*. Here, we validate for the first time the Italian version of MMAS-8 in the evaluation of LT4 treatment adherence in PH. Up to now, MMAS-8 could be considered a new reliable tool that could be further applicable both in clinical and research practice in the setting of levothyroxine therapy, although a validation in other languages is necessary to allow an international use. However, event though the validation of the MMAS-8 is acquired, a significant relationship between the 8-items score to TSH serum levels were not found. Thus, we are not able to use the MMAS-8 score to predict the response to LT4 treatment, even if several pitfalls should be considered. First of all, the TSH serum levels change slowly *(Wiersinga 2001)*, whereas the mean follow-up interval was around four months in our study. Second, the adherence to LT4 therapy does not necessarily reflect TSH serum levels, which, in turn, could be influenced by several other factors, such as concomitant medication influencing the adsorption and the LT4 dose used. This latter represents the most important challenge in the evaluation of MMAS-8 score and TSH relationship, considering the difficulty to find a precise LT4 dose applicable to all patients, as discussed above.

However, a subdivision of patients in low, medium and highly adherent categories was previously suggested *(Morisky 2008)*. Although this classification is useful in our cohort of patients, no differences in TSH serum levels were found, confirming that other parameters, besides adherence, are able to influence TSH modifications. However, we found that highly adherent patients were older and treated with at least 3 concomitant medications than low adherents. These results suggest that adherence to LT4 treatment

is influenced by age and the presence of concomitant medications, whereas no correlation was found with sex, the complexity of the LT4 regimen chosen, the LT4 formulation and the time to follow-up. Thus, specific studies should be drawn to better describe the factors influencing the LT4 adherence.

In 43% of cases, hypothyroidism was discovered incidentally, whereas the majority of remaining patients underwent TSH examination because of suggestive symptoms. Thus, hypothyroidism in Italy is generally detected before the onset of clinical symptoms, allowing the start of appropriate management, limiting the occurrence of overt hypothyroidism. Moreover, chronic autoimmune thyroiditis was found in about half of our cohort. This result is in line with the previous survey in the French population *(Delemer 2012)*, confirming that in iodine-deficient areas this represents the most frequent cause *(Vanderpump 1995)*. The majority of our patients (64%) showed a subclinical hypothyroidism, with a prevalence of a mild (87%) rather than a severe form. This finding appeared different compared to previously data acquired in the French population, in which subclinical hypothyroidism prevalence was about 35% *(Delemer 2012)*. Moreover, a different gender incidence of the disease is discovered, although the management was similar between males and females. Despite the observational design of this trial, the short time-interval chosen for the observation could represent a limitation, considering the chronic feature of the natural history of hypothyroidism.

Conclusion

The EDIPO study provides a valuable snapshot of the approach to clinical management of hypothyroidism in Italy. The wide number of Italian endocrinology centers enrolled and of hypothyroid patients included makes our findings robust and probably representative of the entire Italian population. This study showed different approaches to define the LT4 starting dose by the clinicians, confirming the weight of personalization in chronic treatment. Thus, specific studies are needed to better establish the correct LT4 starting dose. The definition of a specific algorithm could be considered in order to detect the correct dose, connecting all factors known to influence the response to hypothyroidism treatment. In line with this, a high adherence of endocrinologists to both American and European guidelines is demonstrated, with the exception of the LT4 starting dose. Finally,

it is the first time the MMAS-8 questionnaire was used in patients with hypothyroidism. We validated the Italian version of the MMAS-8 questionnaire for the adherence to LT4 treatment. Although the final score does not predict the TSH response to the therapy, the subdivision in low, medium and high adherents patients is helpful to better understand new factors influencing the treatment adherence.

Tables

Table 1. Characteristics of treatment chosen and hormones between baseline visits and follow-up.

	Baseline	**Follow-up visit**	**p-value**
TSH (mIU/mL)	12.54±20.95	4.57±7.54	**<0.001**
Free T4 (pg/mL)	8.72±3.14	10.54±8.82	**<0.001**
Free T3 (pg/mL)	3.19±1.03	3.05±0.99	0.533
TgAbs measurement	568 (54.7)	297 (22.7)	-
TPOAbs measurement	658 (63.3)	297 (22.7)	-
Autoimmune thyroiditis	482 (46.4)	-	-
Thyroiditis at ultrasonography	660 (63.5)	-	-
Nodular goiter	268 (25.8)	-	-
Therapy			
LT4 started	709 (76.1)	-	-
LT4 dose changed	-	143 (16.3)	-
LT4 formulation chosen in the entire cohort of patients			
Tablets	614 (76.2)	-	-
Liquid formulation	114 (14.1)	-	-
Drops	6 (5.3)	-	-
Vials	108 (94.7)	-	-
Soft gel capsules	78 (9.7)	-	-

Footnotes to table 1: vials indicate a LT4 liquid formulation packaged in ampoules.

Table 2. Concordance to AACE/ATA guidelines.

	Recommendation n.	Recommendation	Survey Concordance (%)
Diagnosis	R1	TPOAb measurement should be considered in subclinical hypothyroidism	71.7
	R10	T3 or fT3 should not be use to diagnose hypothyroidism	100
Therapy	R22.1	Use LT4 oral mono-therapy	99.6
	R22.2	Do not use LT4/LT3 combinations to treat hypothyroidism	99.6
	R22.4	Do not use desiccated thyroid hormone to treat hypothyroidism	100
	R32.1	Do not use iodine supplementation in iodine-sufficient areas	99.5
	R33	Selenium should not be used to prevent or treat hypothyroidism	89.6
	R15	Patients with TSH above 10 mIU/L should be considered for treatment with LT4	95.9
	R23	LT4 should be taken on an empty stomach: with water 30–60 minutes before breakfast or at bedtime 4 hours after the last meal. Concomitant intake of substances or medications interfering with its absorption should be avoided.	98.4
LT4 dose	R22.7.1	Young adult patients with overt hypothyroidism should be treated with a full replacement dose (1.6 μg/kg daily)	5.3
	R.22.7.2	Patients > 50 years with overt hypothyroidism and without coronary disease: 50 μg daily.	43.2
	R 22.8	Patients with subclinical hypothyroidism, initial LT4 dose is generally lower: 25–75 μg, depending on the degree of TSH elevation.	80.2
	R.22.7.2	Gradually restore euthyroidism in patients older than 50-60 years of age	53.4
Follow-up	R8	Measure both TSH and fT4 to monitor LT4 treatment	100
	R13	Measure TSH 4-8 weeks after starting or adjusting LT4	20.3

fT3: free triiodothyronine; fT4: free thyroxine; LT3: liothyronine; LT4: levothyroxine; n: number; T3: triiodothyrodinine; TPOAb: antithyroid peroxidase antibodies; TSH: serum thyroid-stimulating hormone

Table 3. Concordance with ETA guidelines.

	Recommendation n.	Recommendation	Survey concordance (%)
Diagnosis	1	Measure TPOAbs	71.7
Therapy	16	Use monotherapy with oral LT4	100
	16	Do not use LT3 alone or LT4 and LT3 combinations to treat hypothyroidism	100
	7	Treat patients with persistent subclinical hypothyroidism and diffuse or nodular goiter to normalize TSH levels	77.4
	13	LT4 is recommended in younger patients, with serum TSH >10 mIU/L, with or without symptoms	92.6
	19	LT4 should be taken on an empty stomach, in the morning, an hour before food, or at bedtime, 2 h or more after the last food. Avoid or take 4 h or more after its ingestion medications interfering with LT4 absorption	97.8
LT4 dose	17	For patients without cardiac disease, a dose of 1.5 μg/kg daily should be use	12.5
	18	For patients with cardiac disease: 25 or 50 μg daily	83.3
	18	In the elderly: 25 or 50 μg daily	77.8
	18	For patients with cardiac disease and in the elderly: increase the dose of LT4 by 25 μg daily every 14–21 days until a full replacement dose is reached	8.3
Follow-up	20	Measure TSH 2 months after starting LT4	67.7

fT3: free triiodothyronine; fT4: free thyroxine; LT3: liothyronine; LT4: levothyroxine; n: number; TPOAb: antithyroid peroxidase antibodies; TSH: serum thyroid-stimulating hormone.

Table 4. Morisky Medical Adherence Scale (MMAS)-8 inter-items variability, evaluated by Cronbach coefficient alpha. The Italian version of the questions and the respective translation is reported.

Item	**Cronbach Coefficient alpha**
1. Do you ever forget to take medications for hypothyroidism? *(Le capita mai di dimenticare di prendere i farmaci per l'ipotiroidismo?)*	**0.540**
2. Sometimes people do not take the drugs for reasons other than forgetfulness. In the last two weeks, there have been days when you did not take the drugs for hypothyroidism? *(A volte le persone non prendono i farmaci per motivi diversi dalla dimenticanza. In riferimento alle ultime due settimane, ci sono stati giorni in cui non ha preso i farmaci per l'ipotiroidismo?)*	**0.577**
3. Have you ever reduced or stopped taking the drugs without telling the doctor, taking them because you felt worse? *(Ha mai ridotto o interrotto l'assunzione dei farmaci senza comunicarlo al medico, perché prendendoli si sentiva peggio?)*	**0.613**
4. Do you ever forget to take your medications for hypothyroidism when they are away from home or traveling? *(Le capita mai di dimenticare di portarsi i farmaci per l'ipotiroidismo quando è fuori casa o in viaggio?)*	**0.572**
5. Yesterday took medication for hypothyroidism? *(Ieri ha preso i farmaci per l'ipotiroidismo?)*	**0.612**
6. Do you ever stop taking the medication when you consider that hypothyroidism is under control? *(Le capita mai di interrompere l'assunzione dei farmaci quando ritiene che l'ipotiroidismo sia sotto controllo?)*	**0.587**
7. For some people take daily medications is a real hassle. Do you ever resent the fact that they have to stick to the treatment plan for hypothyroidism? *(Per alcune persone prendere ogni giorno i farmaci è una vera seccatura. Le capita mai di provare fastidio per il fatto di doversi attenere al piano terapeutico per l'ipotiroidismo?)*	**0.550**
8. How often do you have trouble remembering to take any medications? *(Quanto spesso ha difficoltà a ricordare di prendere tutti i farmaci?)*	**0.576**

Cronbach coefficient alpha was considered significant in each item when it was greater than 0.3.

Table 5. Characteristic of participants who fulfil the Morisky Medical Adherence Scale (MMAS)-8 score. Data are expressed as mean±SD. MMAS-8 Score: low adherence <6; medium adherence from 6 to 8; high adherence >8. The p-value referred to the differences among three categories of adherence.

	Total (n=293)	**High adherent (n=110)**	**Medium adherent (n=113)**	**Low adherent (n=70)**	**p**
Age (years)	47.54±14.97	50±14.09	47.112±15.55	44.34±14.92	**0.036**
Gender					
• Male: n (%)	42 (14.3)	14 (12.7)	17 (15)	11 (15.7)	0.887
• Female: n (%)	251 (85.7)	96 (87.3)	96 (85)	59 (84.3)	0.887
Formulation					
• tablets	234 (79.7)	81 (75.0)	95 (84.1)	58 (80.0)	0.131
• liquid	38 (13.1)	18 (16.7)	14 (12.4)	6 (8.6)	0.131
• soft gel capsules	21 (7.2)	9 (8.3)	4 (3.5)	8 (11.4)	0.131
Weekly regimen					
• stable	280 (96.2)	103 (95.4)	109 (95.4)	68 (97.1)	0.561
• variable	11 (3.8)	5 (4.6)	4 (3.5)	2 (2.9)	0.561
Concomitant medications: n (%)					
0	181 (61.8)	71 (64.6)	65 (57.5)	45 (64.3)	0.117
1	57 (19.4)	14 (12.7)	28 (24.8)	15 (21.5)	**0.021**
2	24 (8.2)	10 (9.1)	9 (8)	5 (7.1)	0.117
≥3	31 (10.6)	15 (13.6)	11 (9.7)	5 (7.1)	**0.016**
Serum TSH					
• mean (microIU/L)	4.55±8.13	4.56±5.74	5.03±11.30	3.76±4.36	0.139
• within range: n (%)	186 (63.3)	69 (62.7)	72 (63.7)	45 (64.3)	0.947
MMAS-8 score	6.72±1.42	8±0.00	6.76±0.50	4.62±1.05	**<0.001**

Footnotes to table 5: n: number; TSH: serum thyroid-stimulating hormone.

THYROID CANCER PHENOTYPES IN RELATION TO METABOLIC SETTING AND RISK FACTORS

Introduction

Most epidemiological studies concerning differentiated thyroid cancers (DTC) indicate an increasing incidence over the last two decades. This increase might be partially explained by the better access to health services worldwide, but clinicopathological analyses do not fully support this hypothesis, indicating that there are carcinogenetic factors behind this noticeable increasing incidence *(Marcello 2014)*. Environmental/lifestyle factors (e.g., radiation, iodine intake, and nitrates), insulin resistance, inflammation, and perhaps a complex multiplication of these factors are considered possible causes for a true increase in thyroid cancer incidence *(Yihao 2017).*

Radiation Exposure. After the nuclear power accident of Chernobyl in 1986, a large increase in childhood thyroid cancer incidence was reported in contaminated areas *(Williams 2008)* and after acute exposure before age 20, the excess relative risk of thyroid cancer has been found to persist for over 50 years *(Furukawa 2013)*. Radiation-induced papillary thyroid cancer (PTC) is unique in its molecular profile that most cases were found to have RET-PTC chromosomal rearrangements, while BRAF or RAS point mutations, commonly found in sporadic PTCs, rarely occurred *(Williams 2008)*. While radiation exposure may have contributed a few extra cases of thyroid cancer diagnosed every year, it could not have explained the sharp increase in thyroid cancer incidence.

Iodine Intake. Iodine is an essential element for the synthesis of thyroid hormones. Since universal salt iodization was introduced in the 1990s, whether iodine contributes to the increasing cancer incidence has been a topic of controversy and public concern.
Level of iodine intake affects thyroid functions, but mechanisms linking with thyroid cancer are not clear. Chronic stimulation of the thyroid-stimulating hormone (TSH) and BRAF mutations in PTC are possible pathways *(Fuziwara 2014, Guan 2009)*. Also, some

studies suggested that iodine intake may influence the distribution of thyroid cancer subtype, rather than the overall incidence. There may be more follicular and fewer papillary carcinomas in iodine-deficient areas and more papillary subtype in iodine-rich areas *(Bubenhofer 1977, Williams 1977)*.

Obesity, Diabetes and Insulin Resistance. The trend of rising thyroid cancer incidence over the past few decades also coincides with the growing trend of obesity and diabetes *(Schmid 2013)*, but whether or how they are correlated is largely unknown. Mechanisms for a possible link between obesity, diabetes, and thyroid cancer include elevated levels of insulin resistance and TSH. Insulin resistance may activate insulin and the IGF pathway, which are important to cell proliferation and apoptosis *(Aschebrook-Kilfoy 2011, Pazaitou-Panayiotou 2013)*. The chronically elevated circulating insulin levels may influence thyroid cancer risk mediated by insulin receptors overexpressed by cancer cells. However, the specific effects of hyperinsulinemia and insulin resistance on promoting thyroid cancer risk are not well understood.

Estrogen and Reproductive Factors. Estrogen has historically been proposed as a potential mechanism mediating the risks of different types of cancer *(Santen 2015, Ashton 2009, Gallo 2010)*. Given that women account for over three-fourth of the prevalence of thyroid cancer, estrogen is regarded as a possible risk factor *(Magri 2012)*. The exposure to exogenous estrogen has also seen an increasing trend due to various medical and environmental sources. Evidence linking these to thyroid cancer risk is, however, nonconclusive. Cellular studies have shown that estrogen and its receptors play an important role in the proliferation, migration, and invasion of thyroid cancer *(Kamat 2011, Rajoria 2011, Rajora 2010)*. It exerts its growth-promoting effect via a membrane-bound estrogen receptor (ER) *(Lu 2016)*. However, such effects or pathways have not been successfully demonstrated in human studies.

Hashimoto's Thyroiditis and inflammation. The incidence of chronic autoimmune Hashimoto's thyroiditis (HT) has increased in the past two decades, paralleling the trend of increased thyroid cancer incidence *(Lun 2013)*. Yet the link between HT and PTC has long been a topic of controversy. Some plausible mechanisms have been proposed.

Elevated levels of TSH found in hypothyroid patients with autoimmune thyroid disease may stimulate follicular epithelial proliferation, promoting the development of papillary carcinoma. Autoimmune thyroiditis might also induce thyroid tumorigenesis via the production of proinflammatory cytokines and oxidative stress *(Khatami 2009)*.

This section aims to explore the role of metabolic setting and other risks factor in determining thyroid tumorigenesis and thyroid cancer phenotypes, in particular:

- the clinical and histopathological characteristics of 2 Italian populations with DTC exposed to different risk factors, in order to evaluate the differences in DTC phenotype.
- the role of inflammation and autoimmunity in determining the thyroid cancer phenotype
- the relationship between insulin resistance, adipokines levels and the presence of DTC

How do etiological factors can explain the different clinical features of patients with differentiated thyroid cancer and their histopathological findings?

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Abstract

The aim was to retrospectively analyse the clinical-histopathological characteristics of patients with newly diagnosis of differentiated thyroid cancer (DTC) referred to two Italian centres, one in Northern and the other in Southern Italy, between 2000 and 2013. 1081 patients were included and subdivided into two groups: group A (474 patients from Novara) and group B (607 patients from Naples). The group A came from the industrial area of Novara, while the Group B came from the areas around Vesuvius and Campi Flegrei. The two groups were comparable for iodine levels, body mass index, diagnostic timing and clinical procedures. For all patients, demographic and clinical data were collected. No difference was found in gender, whereas the age at diagnosis was later in the group A (group A 53.1±15.16 years, group B 41.9±14.25 years, $p<0.001$). In both groups, the most frequent histotype was papillary thyroid cancer (PTC) with prevalence of follicular variant in group A ($p<0.0001$) and classical variant in group B ($p<0.0001$). Aggressive histological features were mainly seen in group A (bilaterality $p<0.0001$, multifocality $p<0.0001$ and thyroid capsular invasion $p<0.0001$). Microcarcinomas were more frequent in group A ($p<0.0001$) but mostly characterized by bilaterality ($p<0.001$) and multifocality ($p<0.04$). In both groups, tumour-associated thyroiditis showed a significant increase over the years (group A $p<0.05$, group B $p<0.04$). Environmental factors could justify the differences found in our study. These preliminary data should stimulate the need for an Italian Cancer Registry of DTC in order to allow an epidemiological characterization, allowing the identification of specific etiological factors and an improvement in the management of the disease.

Background

The incidence of the differentiated thyroid carcinoma (DTC) has been increasing in many countries over the last 30 years *(Howlader 2013, Davies 2014, Kent 2007, Simard 2012, Enewold 2009, Ascherbrook-Kilfoy 2011, Colonna 2007, Reynolds 2005, Netea-Maier 2008, Lise 2012, Elisei 2010)*.

The most recent data about this massive increase in the incidence come from the U.S. SEER (Surveillance, Epidemiology, and End Results) which shows that the age- adjusted incidence rate has increased from 4.85/100,000/year in 1975 to 14.93/100,000/year in 2011 *(Howlader 2013)*.

In Italy, a marked increase occurred over the last three decades, both in men (+9.1% per year) and in women (+8.7% per year), according to AIRTUM (Italian Association of Cancer Registries), AIOM data (Italian Association of Medical Oncology) and literature *(Elisei 2011)*; in particular, more than 16,000 new cases were seen in 2013 (4100 men and 12,200 women), representing about 4.5 % of all new cases *(AIRTUM 2013)*.

The reported incidence increase in all countries is almost exclusively due to an increase in the papillary thyroid carcinomas variant (PTC), with no significant changes in the follicular (FTC), medullary and anaplastic form *(Davies 2014)*. Despite the most part of PTC consists in indolent micro/small carcinomas *(Davies 2014)*, the mortality rate has not decreased, standing around 0.5 per 100,000 per year in both sexes in almost all countries *(Ferlay 2004, Ferlay 2010)*.

Therefore, even if the increased incidence could be attributed to a better diagnostic accuracy *(Elisei 2010, Lee 2012, Grodski 2008)*, we cannot exclude environmental and lifestyle causative factors *(Pellegriti 2013, Morris 2010, Li 2013)*.

Major data about epidemiology and risk factors for DTC come from geographical areas, such as United States, where the vastness, the presence of different ethnic groups and different lifestyles make difficult to evaluate the environmental carcinogens *(Davies 2014, Simard 2012, Enewold 2009, Ascherbrook-Kilfoy 2011)*. In Italy, this limit could be avoided thanks to the territorial limited extension, the more homogeneous population and the comparable lifestyle in all regions *(Malandrino 2013, Pellegriti 2009, Biondi 2012, Marcello 2014)*.

The aim of our study was to retrospectively analyse the clinical and histopathological characteristics of Italian caucasic patients with DTC referred to two Italian centres, the University Hospital (AOU) “Maggiore della Carità” in Novara, in Northern Italy and the University Hospital (AOU), Federico II in Naples, in Southern Italy, in order to evaluate the possible differences of DTC in these populations compared to data reported in the national literature.

Methods

We retrospectively analysed clinical and pathological features of patients with newly diagnosis of DTC referred to 2 clinical centres: “Maggiore della Carità” University Hospital (Novara) in Northern Italy and “Federico II” University Hospital (Naples) in Southern Italy, from January 2000 to December 2013. All cases were collected from the Pathology archive of each centre.

Patients were subdivided into two groups, according to the centre of origin (Group A=Novara; Group B=Naples). The group A patients came from the eastern province of Novara, a typical industrial area, while the Group B population came from the areas around the volcano Vesuvius and Campi Flegrei, an active sunken volcanic area.

According to ARPA (Regional Agency for Environmental Protection) data, there are some differences in exposure between these two Italian regions: Novara population is exposed to industrial chemicals, such as formaldehyde and benzene *(ARPA Piedmont 2014)*, while Naples area is exposed to minerals of volcanic origin such as nitrates, vanadium, manganese and other heavy metals *(ARPA Campania 2014)*.

The two groups were comparable for iodine levels, body mass index (BMI), diagnostic timing and clinical procedures.

Patients data were collected through retrospective medical record review regarding demographics and clinical aspects (age, gender, BMI, ioduria, type of surgery) and histological reports including information about tumour size, extrathyroidal extension, multifocality, lymph-nodal (LN) metastasis and presence of peritumoural thyroiditis.

Environmental and occupational exposures were identified through a direct interview for each patient. Smoking habits, hormonal and genetic factors were not investigated because the evaluation of this clinical outcome was not a purpose of the study.

Patients with history of medical irradiation, poorly differentiated, anaplastic, medullary thyroid carcinomas and secondary tumours or living for less than 5 years in the analysed areas were excluded.

Our data relating to ioduria were extrapolated from a new document, which contains information about ioduria in 16 Italian regions including Piedmont and Campania. This document was published by Italian "Istituto Superiore di Sanità" in 2014 *(ISS 2014)*.

Histological diagnoses were blindly determined by two independent pathologists, specialized in thyroid pathology, belonging to the two centres. Pathologic staging was redefined according to the Tumour, Lymph Node, and Metastasis classification system based on the 6th edition of the UICC/AJCC TNM classification (Edge 2010).

Moreover, we evaluated the frequency and histopathological characteristics of microcarcinomas (defined as tumours 1 cm or less in size *(LiVolsi 2004)*) in both the groups.

For all cases, tumour-associated thyroiditis was assessed. The histological criteria used to make this diagnosis included diffuse lymphoplasmacytic infiltration, germinal centres and enlarged epithelial cells with large nuclei and eosinophilic cytoplasm (Hurthle cells), which are specific for Hashimoto's thyroiditis (HT) and also polynuclear macrophage-like and fibroblastic-like population specific for non-specific lymphocytic thyroiditis, which might represent perineoplastic inflammation, when occurring immediately adjacent to a tumour *(Liotti 2012)*.

The results were expressed as mean ± standard deviation (SD) or percentage with 95 % confidence intervals. The association between factors was assessed with the multinomial logistic regression for dichotomous or categorical variables. Continuous variables were analysed using student t test.

P values less than 0.05 were considered significant. The analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, Ill., USA).

Results

1081 patients were included in this study: group A=474 patients and group B=607 patients. The clinical and histopathological characteristics of all patients are reported in Table 1 (pag. 158).

Group A (474 cases)

In this population, the majority of patients were female (76.4 %) with a M:F ratio of 1:3. The mean age at diagnosis (mean±SD) was 53.1±15.16 years (range: 13.0–88.0 years); in particular, 68.6% was aged >45 years. The mean BMI (mean±SD) was 26.6±4.52 kg/m^2.
Total thyroidectomy was performed in 99.2% of cases and 28.1% of patients also underwent lymphadenectomy. The most frequent histotype was PTC (90.0%), and the most widespread variant was the follicular one (37.3% of all PTCs), followed by the classical variant (36.9%). FTC was detected in 9.0% of cases. Only 5.5% of tumours were sized >4.0 cm, while microcarcinomas represented the majority of cases (44.1%).
The 75.3% of tumours were classified as stage I, followed by stage III tumours (11.4%), with high frequency of thyroid capsular invasion (19.2%). Tumours were bilateral in the 25.3% of cases and multifocal in the 31.2% of cases. Among patients who underwent lymphadenectomy, 50.8% had lymph node metastasis.
Finally, tumour-associated thyroiditis was histologically found in 24.5% of cases.

Group B (607 cases)

In this group, the disease was more frequent in females with M:F ratio of 1:3.
The mean age at diagnosis (mean ± SD) was 41.9±14.28 years (range: 8.0-84.0 years); in particular, 58.3% of the patients were aged <45 years. The mean BMI (mean±SD) was 27.1±5.57 kg/m^2.
99.7% of patients underwent total thyroidectomy with lymphadenectomy in 46.9% of cases. The most frequent histotype was PTC (89.0%), and the most widespread variant was the classical (63.8% of total PTCs), followed by follicular (19.9%). The 11.0% of patients was affected by FTC.

Tumours sized >1.0 cm and <2.0 cm represented the majority of cases (43.9% of cases).
Stage I tumours represented more than three-quarters of the cases (88.4%), followed by stage II (5.5%), stage III (4.1%) and stage IV (2.0%) tumours.
Tumours were bilateral in 13.2% of cases and multifocal in 21.1% of cases, and the thyroid capsular invasion was found only in 8.7% of cases. Among patients who underwent lymphadenectomy, 50.2% had lymph node metastasis.
Finally, the tumour-associated thyroiditis was histologically found in 29.7% of cases.

Group A vs Group B

With regard to the clinical characteristics, the age at diagnosis was earlier in group B than in group A ($p<0.001$) (Figure 1, pag. 159).
Moreover, no significant difference was found in BMI (26.6±4.52 kg/m^2 in group A and 27.1±5.57 kg/m^2 in group B).
In both populations, we observed an upward trend over time of PTC cases (Figure 2, pag. 159). Regarding histotype, the most frequent was PTC in both the groups; the follicular variant was more represented in group A, while the classical variant in group B ($p<0.0001$) (Figure 3, pag. 159). Furthermore, most aggressive variants were observed more in group A than in group B, although without statistical significance. Histopathological differences were found between the two populations: bilaterality (group A vs. group B, 25.3% vs. 13.2%, $p<0.0001$), multifocality (group A vs. group B, 31.2% vs. 21.1%, $p<0.0001$) and thyroid capsular invasion (group A vs. group B, 19.2% vs. 8.7%, $p<0.0001$) (Figure 4, pag. 160).
Regarding smaller tumours, microcarcinomas were significantly more frequent in group A ($p<0.0001$) with respect to group B, and, similarly to macroscopic tumours, in Novara's group they were mostly characterized by bilaterality (group A vs. group B, 20.5% vs. 8.3%, $p<0.001$), multifocality (group A vs. group B, 25.9% vs. 16.6%, $p<0.04$) and, without reaching significance, thyroid capsular invasion (group A vs. group B, 6.3 % vs. 4.1 %).
Therefore, stage I tumours were more represented in group B than in group A ($p<0.0001$), considering the earliest age of discovery of the disease (Figure 5, pag. 160).
With regard to the presence of lymph node metastasism (N) and distance metastasis (M) at the time of diagnosis, no difference was found between the two groups, nor about the

presence of thyroiditis, considering both thyroid peroxidase antibodies (TPOAb) positivity and the presence of lymphocytic and non-specific lymphocytic peritumoral inflammation; however, this tumour-associated thyroiditis showed a significant progressive increase over the years, in both the populations ($p<0.05$ in group A, $p<0.04$ in group B) (Figure 6, pag. 160).

Discussion

The study of clinical, histopathological and epidemiological characteristics directly influences diagnostic and therapeutic decisions in the field of oncology *(Sipos 2010)*.

In recent years, the literature reported a significant increase in the incidence of PTC worldwide and in particular in Italy *(Howlader 2013, Davies 2014, Kent 2007, Simard 2012, Enewold 2009, Ascherbrook-Kilfoy 2011, Colonna 2007, Reynolds 2005, Netea-Maier 2008, Lise 2012, Elisei 2010)*.

In our study, we observed the same upward trend in incidence of thyroid cancer cases in both the cohorts studied; however, considering the clinical and histopathological characteristics at the time of diagnosis, some differences emerge, leading to hypothesize that environmental and carcinogens factors could change the DTC tumourigenesis.

First, we observed a difference in age at diagnosis between the two populations. In fact, in the Neapolitan population, thyroid cancer seems to be diagnosed earlier than in Novara population, in agreement with other national and international studies reported in literature *(Elisei 2010, Shi 2016)*. Interestingly, in group B, we observed a high percentage of subjects younger than 25 years. This anticipation in the age at diagnosis was also found in the Italian study by Malandrino and co-workers *(Malandrino 2013)* involving populations living in volcanic areas. As a matter of fact, Neapolitan patients mainly came from the areas around the volcano Vesuvius and Campi Flegrei, which are exposed to volcanic minerals, according to ARPA data *(ARPA Campania 2014)*. Therefore, this phenomenon of age anticipation could be related to the exposure to one or more volcanic carcinogens (in particular heavy metals) *(Pellegriti 2009, Biondi 2012, Marcello 2014, Duntas 2009, Arnbjornsson 1986, Kolonel 1990, Curado 2007, Truong 2007)*. It has already been reported that high heavy metal concentration in the soil or water would act as endocrine disruptors, increasing the risk for thyroid cancer in younger age because

their effects may manifest in utero, in children and in adulthood *(Patrick 2009)*. Moreover, considering only thyroid cancers larger than 1.0 cm, in this group, we found a significantly higher incidence of larger thyroid cancers, even papillary types, according to other studies regarding populations living in volcanic areas *(Pellegriti 2009, Truong 2007)*. In these areas, there seems to be a significant inverse relationship between tumour size and age at diagnosis (the mean tumour size was significantly higher in younger patients in a Sicilian cohort around Etna volcano) *(Malandrino 2013)*.

On the other hand, in Novara's group, we found older age at diagnosis, differently from what reported in literature *(Davies 2014, Elisei 2010, Shi 2016)*. It has to be considered that also Fugazzola and co-workers (Abstract no. 20. Abstract book from 9° Congresso Associazione Italiana della Tiroide) recently showed an increased age at diagnosis in their population living in Milan and surrounding areas, close to Novara region.

This increase in thyroid cancer incidence in elderly population could be due to the combined effect of radiations and natural changing of hormonal status in this age *(Fuzik 2013, Prysyazhnyuk 2014)*. Therefore, considering that Northern Italy was more exposed to Chernobyl's nuclear radiations than Southern Italy, as shown in the European map from Chernobyl, 22 years later, CBS News *(Plante 2008)*, we could speculate that in Novara's group, we are now observing the second peak of tumours after about 20 years of latency.

Another interesting observation coming from our data concerns the histopathological features. In Novara's group, we found a higher percentage of follicular variant of PTC (FVPTC) compared to Neapolitan group and other Italian cohorts *(De Leo 2013)*. The FVPTC is a heterogeneous disease that includes two different groups of tumours: one similar to the classical PTC in its behaviour and another more similar to FTC in its invasive features and molecular profile *(Liu 2006)*. Certainly, the re-classification of FVPTC in these 2 subgroups and the genetic characterization of tumours in our population might be helpful to explain their higher incidence in Novara's group.

Furthermore, in Novara's cohort, CDT showed more histopathological aggressive features already at diagnosis, such as thyroid capsular invasion, bilaterality and multifocality. The same was seen in microcarcinomas, especially with regard to bilaterality and multifocality, even if in literature these two characteristics do not seem to adversely affect survival *(Elisei 2010)*. This result corroborates the data available in the literature

(Tzvetov 2009) showing that microscopic thyroid cancer at presentation can be as aggressive as macroscopic disease, regardless of whether the tumour is incidentally discovered or not. Molecular studies would be able to differentiate, at an early stage, microcarcinomas with an aggressive behaviour from microcarcinomas with an indolent clinical course. The increased aggressiveness could correlate with and another risk factor, of which Novara's population is exposed, chemical pollutants in atmosphere *(Fincham 2000, Carstensen 1990)*. As a matter of fact, Novara is placed in a predominantly industrial area and the population is exposed to chemical pollutants such as formaldehyde and benzene *(ARPA Piedmont 2014)*. Most of the population came from the Eastern part of the Novara province, where the industries are predominantly placed, especially in the textile and engineering fields; about 30.0% of our patients were employed in the textile sector and about 25.0% in the engineering industry. According to ARPA data collected from 2003 to 2009 *(ARPA Piedmon 2014)*, environmental exposure to formaldehyde and benzene in this area was recognized to be higher than in the rest of Italy. The correlation between thyroid cancer and exposure to formaldehyde and benzene emerges in several studies reported in literature *(Wong 2006, Kuzmickiene 2009, Lope 2009)*. In particular, a study conducted on a cohort of Chinese women working in the textile industry in Shanghai showed a strong association between thyroid cancer and exposure to formaldehyde *(Wong 2006)*. Even exposure to benzene has been shown to be related to thyroid cancer, but with a lower extent than formaldehyde. To date, few studies focused on the role of these substances in thyroid cancer and on the mechanisms of inducing tumourigenesis, but all agree with the hypothesis that these chemicals can determine both the tumour onset and its more aggressive behaviour *(Wong 2006, Kuzmickiene 2009, Lope 2009)*.

We can speculate that in the Northern area both environmental factors (radiation or chemical pollutants exposure), together or alone might play a role in thyroid tumourigenesis. From a recent review by Marcello and co-workers *(Marcello2014)*, radiation exposure seems to be the cause of a high percentage of RET/PTC rearrangements, while chemical pollutants seem to be associated with BRAF mutations, and more specifically BRAFV600E point mutation. Thus, the study of the underlying biological and molecular pathways in our population could help us to understand which etiopathogenetic hypothesis is most probable.

Finally, no significant difference was found in iodine level, being median urinary iodine 87 lg/L in Naples *(Mazzarella 2009)* and 98 lg/L in Piedmont *(ISS 2014)*, as both populations belong to region with mild iodine deficiency. In both the groups, we found a similar distribution of associated thyroiditis with an increasing trend over time in term of both tumour- associated inflammation and TPOAb positivity. This could be explained by 2 hypothesis: 1) the increase in the iodine intake as a result of programmes of iodine prophylaxis *(Aghini-Lombardi 1997)*; 2) external factors (still unknown) probably triggering both thyroid disorders, inflammation and tumour *(Malandrino 2015)*. However, if the increased frequency of thyroiditis would be an additional factor contributing to the continuous increase in incidence of thyroid cancer, it is still debated *(Latina 2013, Cunha 2011)*.

The present study has some potential limitations. It was retrospective and cases were collected from the Pathology archives, so we cannot supply complete information about the reasons of all the thyroid surgical interventions and about the incidental nature of all microcarcinomas. Moreover, we do not have a control group, and thus, we compared our data with others coming only from Italian studies.

The role of these potential chemicals and environmental factors in inducing thyroid cancer is only conceivable. A greater number of studies are required to classify the role of these substances, in order to accurately understand their physiopathological mechanisms of induction of tumourigenesis and the clinical and histopathological characteristics, they are responsible for.

Conclusions

The more sensitive and widespread diagnostic procedures can only partially justify the progressive increase in DTC. More studies are necessary to investigate the role of environmental factors, which include both volcanic elements and industrial chemical pollutants. In this scenario, a national cancer registry could be a mirror of the different territories and could provide a better understanding of the different risk factors and mechanisms involved in tumourigenesis. It could also allow to carry out comparative studies, through which it would be possible to outline the characteristics of the

populations at risk and then to perform screening programmes and prevention, improving the management of DTC.

Tables

Table 1. Clinical and histopathological characteristics of all patients divided into two study groups: group A (Novara) and group B (Naples).

		Group A (Novara) (474 cases)	Group B (Naples) (607 cases)
Age at diagnosis	< 45 years (%)	31.4	58.3
	≥ 45 years (%)	68.6	41.7
	Mean ± SD (years)	53.1±15.16	41.9±14.28
Gender	Male (%)	23.6	22.2
	Female (%)	76.4	77.8
Body Mass Index (BMI)	Mean ± SD (years)	26.6±4.52	27.1±5.57
Type of surgery	Total or near total thyroidectomy (%)	99.2	99.7
	Lobectomy (%)	0.8	0.3
Lymphadenectomy	No (%)	71.9	53.1
	Yes (%)	28.1	46.9
Histotype	PTC (%)	90.0	89.0
	FTC (%)	9.0	11.0
	PTC + FTC (%)	1.0	0.0
PTC variant	Classic (%)	36.9	63.8
	Follicular (%)	37.3	19.9
	Classic and Follicular (%)	9.1	7.6
	Other (%)	16.7	8.7
Size	< 1.0 cm (%)	44.1	30.4
	2.0 cm (%)	27.8	43.9
	2.0-4.0 cm (%)	22.6	21.9
	> 4.0 cm (%)	5.5	3.8
Multifocality	Absent (%)	68.8	78.9
	Present (%)	31.2	21.1
Bilaterality	Absent (%)	74.7	86.8
	Present (%)	25.3	13.2
Capsular Invasion	Absent (%)	80.0	91.3
	Present (%)	19.2	8.7
Staging (TNM 2010)	Stage I (%)	75.3	88.4
	Stage II (%)	8.9	5.5
	Stage III (%)	11.4	4.1
	Stage IV(%)	4.4	2.0
Histological associated thyroiditis	Absent (%)	75.5	70.3
	Present (%)	24.5	29.7
Lymph Node Metastasis	Absent (%)	49.2	49.8
	Present (%)	50.8	50.2
TPOAb[a]	Absent (%)	70.0	67.0
	Present (%)	30.0	33.0

[a]TPOAb assays has a functional sensitivity of 25.0 U/mL and the analysis was automatically performed using ''ADVIA Centaur ATPO'' (Siemens Healthcare Diagnostic inc., Tarrytown, NY, USA)

Figures

Figure 1. Age at diagnosis in groups A and B.

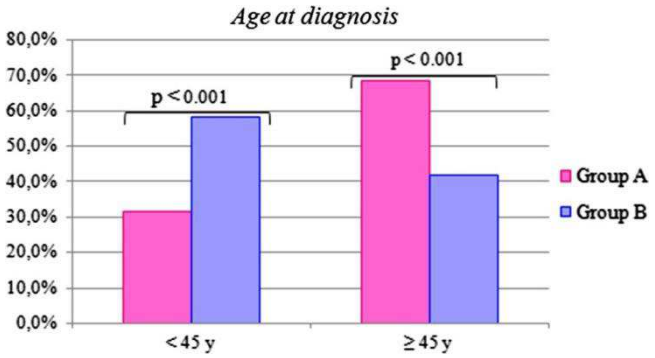


Figure 2. PTC and FTC in groups A and B: trend over the years.

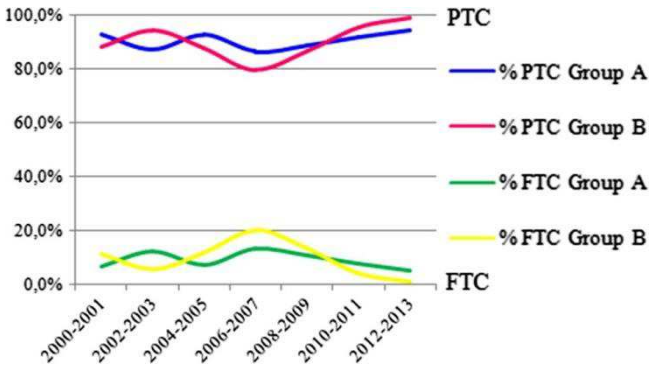


Figure 3. Histotype variants of PTC in groups A and B.

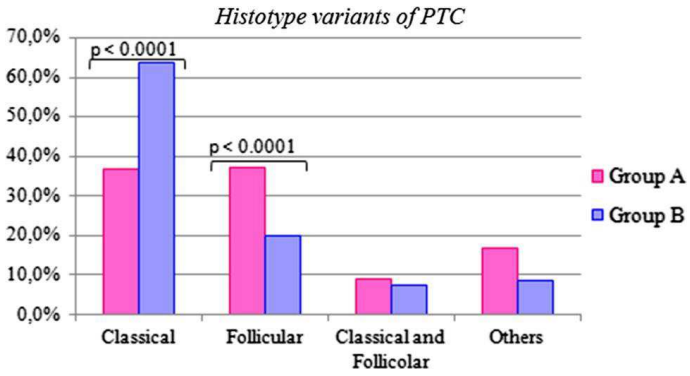


Figure 4. Focality, laterality and thyroid capsular invasion in groups A and B.

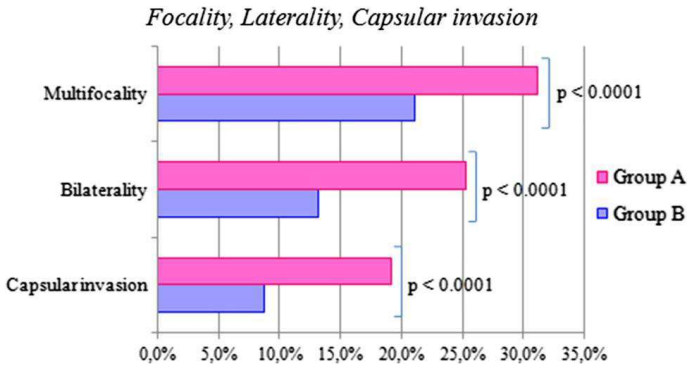


Figure 5. Staging in groups A and B.

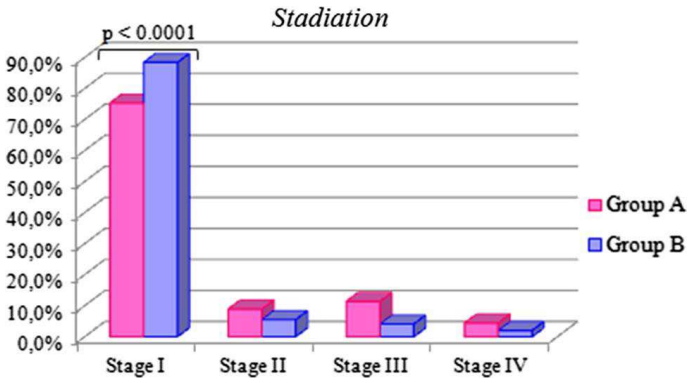
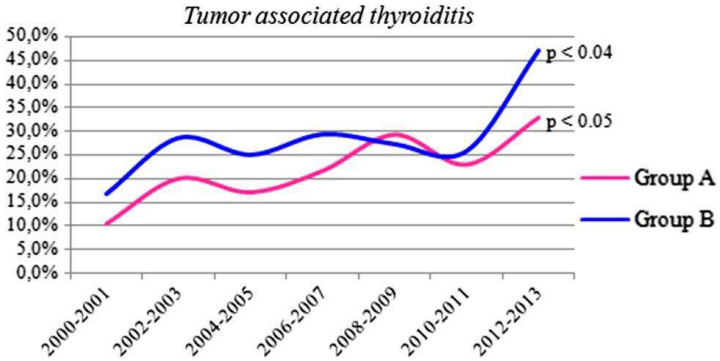


Figure 6. Tumour-associated thyroiditis in groups A and B: trend over the years.



Thyroid cancer phenotypes in relation to inflammation and autoimmunity

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Abstract

Thyroid cancer represents the most frequent endocrine neoplasm and is epidemiologically linked to a growing incidence worldwide, which is only, in part, explained by the increased detection of small cancers in the preclinical stage. Understanding the molecular pathogenesis of well-differentiated and poorly-differentiated thyroid cancers has prompted interest into the identification of crucial signaling pathways and molecular derangements related to genetic and epigenetic alterations. Increasing attention has been recently focused on inflammation and immunity as major culprit mechanisms involved in thyroid tumorigenesis, through the detection of activated immune cells, pro-inflammatory cytokines, as well as signal integrations between inflammatory and proliferative pathways within the thyroid tumor micro-environment (TME). In addition to playing important roles in tumor surveillance and rejection, the presence of tumor-associated macrophages (TAM) and the activation of NF-κB signalling pathway are now reckoned as hallmarks and crucial mediator of inflammation-induced growth and progression of thyroid cancer. Thorough understanding of this immunological link and identification of novel molecular targets could provide unprecedented opportunities for research and development of diagnostic, prognostic and treatment strategies for thyroid cancer.

Background

Thyroid cancer is the most frequent endocrine neoplasia and its incidence rates are on the rise *(Lim 2017)*. Thyroid cancers are usually follicular or para-follicular in their origin, and lesions developing from follicular cells include well differentiated thyroid cancers (DTC), poorly differentiated (PDTC) and anaplastic (ATC) thyroid carcinomas (*Kondo 2006)*. DTCs, which encompass papillary cancers (PTC) and follicular cancers (FTC), usually show a good prognosis after surgery and radioiodine therapy, yet 5–10% of cases progress to radioiodine refractory-disease. On the other hand, PDTCs and ATCs are therapy-resistant and prognosis is unfavorable *(Rosai 1992)*. Medullary thyroid carcinoma (MTC), which originates from the para-follicular C-cells, is either sporadic or familial and, by the time of diagnosis, shows high rate of lymph node metastases, which can elude detection pre-operatively or even intra-operatively *(Wells 2015)*.

The role of inflammation in DTCs has been the focus of several studies published in the last 10 years, which have demonstrated positive association between chronic inflammation and increased risk of developing DTC, and suggested that the inflammatory microenvironment is an essential component of cellular transformation and tumour progression *(Guarino 2010, Melillo 2010, Cunha 2014)*. In support of this inference, there has been demonstration that local activities engaged in thyroid tumorigenesis and thyroid cancer progression are positively influenced by two major inflammatory components: inflammatory cells along with their humoral mediators presenting within the cancer site, and activation of oncoprotein-mediated signalling present in epithelial cancer cells. Inflammatory cells and mediators enrich the tumour stroma and partake in several processes such as tissue remodelling, tissue repair and neo-angiogenesis *(Hanahan 2000)*. Cancer stroma englobes both inflammatory cells engaged in antitumour effects and activated immune cells capable of pro-tumor immune responses, and the balance of antitumour/protumour immune responses culminates in the regulation of cancer suppression vs cancer progression *(Cunha 2014, Poschke 2011)*. Cancer stroma thus plays a dominant role in the development and progression of DTCs *(Modi 2003, Yano 2007, French 2012)*, as well as it shows a potential role in MTC and PDTCs. The aim of this article is to provide an overview on the relationship between inflammation and thyroid cancers in relation to the immune mechanisms regulating thyroid cancer progression. To this

purpose, our at-a-glance description will mainly focus on DTCs, where essential evidences related to different histotypes and relative clinical aspects have been collected, whereas information resumed in MTC and ATC will be presented separately.

Inflammation and DTC

In general, two pathways have been proposed to explain the link between inflammation and DTC *(Mantovani 2008)*: the extrinsic (microenvironment-driven) and the intrinsic (oncogene-driven) pathways.

Extrinsic pathway

Tumour-infiltrating inflammation

The extrinsic pathway is triggered by tumour-infiltrating inflammation that includes leukocyte infiltration, tumour-associated macrophages (TAM), cytokines, chemokines and VEGF *(Liotti 2012)*. TAM and immature dendritic cells accumulate both in tumoral stroma and at the invasive front of PTC, and the strength of their infiltrate positively correlates with capsular invasion, extra-thyroidal extension and aggressive behaviour *(Ryder 2008)*.
According to a classical view, tumours are encircled by extracellular matrix (ECM) with its structural and specialized proteins, as well as by stromal cells, which comprises cancer-associated fibroblasts (CAFs), innate and adaptive immune cells, specialized mesenchymal cells, endothelial cells and pericytes. CAFs impact cancer progression by remodelling the ECM, inducing angiogenesis, recruiting inflammatory cells, and redirecting cancer cell proliferation via secretion of growth factors, immune suppressive cytokines, and mesenchymal-epithelial cell interactions *(Kalluri 2006)*. Cancer stroma contributes to create the so-called tumour microenvironment (TME), which enfolds a significant inflammatory cell infiltrate capable of acting pleiotropically *(Xing 2013)*. While some cells are engaged in antitumour effects (i.e., dendritic cells, tumour infiltrating lymphocytes, M1 macrophages), others instigate a pro-tumour immune response (i.e., neutrophils, mast cells, NK cells, tumour infiltrating lymphocytes, M2 macrophages), such that the (un)balance of antitumour/protumour immune responses intervenes to regulate cancer suppression vs cancer progression *(Cunha 2014, Poschke 2011)*. Per se, TME acts as a

reservoir of pro-tumorigenic and pro-angiogenic cytokines, which are either produced by tumour cells, CAFs, innate or adaptive immune cells. The resulting inflammatory microenvironment is associated with the production of reactive oxygen species and oxidative stress initiated by the transcription of NF-kB and perpetuated by activation of the MAPK pathway *(Xing 2013)*. Several lines of evidence state that TAMs promote cancer cell survival, proliferation, metastases, angiogenesis and immune suppression *(Noy 2014)*. Likely, the impact of TAMs on tumour progression depends on their specific reprogramming within the tumour, a process influenced by factors of the local microenvironment such as hypoxia, locally released mediators (e.i. cytokines, growth factors), as well as metabolic products either released by cancer cells or other immune and stroma-related cells *(Galdiero 2016)*. Pathways and mechanisms involved in TAMs reprogramming are not completely understood, yet activation of the AKT/mTOR pathway has been hypothesized to influence the inflammatory phenotype of DTC-induced macrophages *(Arts 2016)*.

Furthermore, Melillo et al *(Melillo 2010)* demonstrated the complex relationship between mast cells and thyroid cancer cells. PTC displayed an increased density of mast cells with respect to normal tissues, and this would be associated with a worse prognosis. In vitro, papillary thyroid cells recruited mast cells through tumour-derived VEGF-A; in turn, mast cells surrounding the tumour secreted histamine and chemokines (ex. CXCL-1, CXCL10) that induced cancer proliferation, survival and migration by an autocrine and a paracrine loop *(Melillo 2010)*. Likewise, TAMs and mast cells have been experimentally and clinically shown to promote progression of PTC *(Ryder 2013)*.

<u>Obesity-dependent inflammation</u>

Obesity enhances the risk of at least 13 different cancers, and is a risk factor for tumour recurrence after curative surgery, poor survival, non-cancer-related as well as cancer-related mortality *(Steele 2017, Calle 2003)*. Epidemiological linkage between excess body weight and thyroid cancers has been highlighted in cross-sectional studies *(Samanic 2004, Engeland 2006)* and confirmed in meta-analyses of cross-sectional *(Del Maso 2000)* and prospective studies *(Samanic 2004, Engeland 2006, Renehan 2008)*. In a pooled scrutiny of five U.S prospective studies, BMI was associated with thyroid cancer risk in both men and women, independent of tumour histology *(Kitahara 2011)*. Several lines of evidence

suggest a potential role for adipose tissue (AT) accumulation in regulating TME pathophysiology, supported by associations found between obesity-dependent inflammation and cancer *(Marcello 2014)*. As such, there is demonstration that hypoxia, chronic inflammation and oxidative stress, which are typical of obese subjects, could favour the development of a subgroup of DTC characterized by resistance to both 131I treatment and chemotherapy *(Marcello 2014, Ilie 2013, Santini 2014)*. The relevance of AT biology in thyroid tumorigenesis is mainly related to its ability to intervene both as a reservoir and as a regulator of key elements of the immune system, which involve production of immunomodulatory molecules and expression of their receptors within the AT *(Frühbeck 2006, Schäffler 2010)*.

It is known that AT englobes different cell populations, e.g. preadipocytes, mature adipocytes as well as immune and stromal cells. Pre-adipocytes have functional characteristics and transcriptional patterns of multipotent cells that are similar to immune cells, and can transdifferentiate into macrophages both in vitro and in vivo *(Cousin 1999, Charriere 2003)*. Mature adipocytes share the ability to secrete cytokines acting as pro- or anti-inflammatory factors related to AT accumulation. Immune cells englobed in AT include pro-inflammatory T lymphocytes (predominantly CD8+), which contribute to local inflammatory cell activation by attracting macrophages, which perpetuate the inflammatory response within the AT *(Nishimura 2009, Kintscher 2008)*. Both M1 and M2 macrophages can be found in AT *(Haase 2014)*. While resident M2 macrophages play dominant roles in AT physiology and are able to produce anti-inflammatory cytokines, M1-like macrophages are recruited and clustered within the AT as crown-like structures (CLSs) and, upon stimulation by IFNγ or lipopolysaccharide, they are able to produce pro-inflammatory cytokines, thus contributing to inflammatory pathways relating to insulin resistance *(Hill 2014, Cinti 2005, Weisberg 2003, Xu 2003)*. AT hypoxia may promote the M2 to M1 switching *(Fujisaka 2013)*, and these macrophages are responsible for AT expression of TNFα, as well as production of discrete amounts of iNOS and IL6 *(Weisberg 2003)*. TNFα is a cytokine capable of anti-proliferative actions in a human PTC line, through a receptor-mediated mechanism *(Pang 1996)*. Nevertheless, the high TNFα exposure related to obesity seems to induce a state of TNFα resistance, which ultimately facilitates thyroid tumour progression *(Marcello 2014, Pang 1996)* and metastatic diffusion *(Coperchini 2016)*. IL-6 is a cytokine involved in tumorigenesis

(Lumachi 2010), but its role in thyroid cancer is still confusing. Although results linking directly thyroid cancer to IL-6 are scant *(Couto 2012, Chang 2003)*, IL-6 could be important for the inflammation microenvironment in thyroid carcinogenesis, influencing DTC development and progression *(Cunha 2014, Lumachi 2010)*. Recently, Kobawala et al *(Kobawala 2016)* demonstrated that IL-6 mRNA expression is higher in the primary tumour tissues of PTC patients as compared to the corresponding adjacent normal tissues, and that serum IL-6 correlates with larger tumour size, presence of distant metastasis, extra-thyroidal extension and poor overall survival.

The obesity-related adipocytokine network also accounts for the biological activity of AT in relation to the development of thyroid cancer *(Cunha 2014, Marcello 2014, Santini 2014, Dalamaga 2012)*. Mature adipocytes produce leptin, which is involved in activation of monocytes and macrophages, stimulation of VEGF and angiogenesis, and suppression of anti-inflammatory cytokines. Leptin promotes cell migration of PTC, while inhibiting the migration of follicular cells *(Cheng 2010a)*. Studies in vitro demonstrated that leptin is able to stimulate a more aggressive PTC phenotype by activating the PI3K/AKT pathway *(Uddin 2010)*, as well as it promotes the de-differentiation of thyroid cancer cells via the JAK2/STAT3 signalling pathway *(Kim 2013)*. Recently, Fan et al *(Fan 2015)* demonstrated that leptin had negative prognostic significance in PTC, whereas it may play a protective role in FTC.

On the contrary, adiponectin, an adipocytokine with strong anti-inflammatory properties capable of proapoptotic and antiproliferative effects, appears to be inversely correlated with DTCs, suggesting a protective effect against tumorigenesis *(Mitsiades 2011)*. Accordingly, Cheng et al *(Cheng 2013)* found that, when tissues were negative for adiponectin receptors, tumours were significantly associated with extrathyroidal invasion, multicentricity, and higher TNM stage, demonstrating that the expression of adiponectin receptors could associate with a better prognosis. Further potential links between obesity-related inflammation and DTC are represented by ghrelin and obestatin. In particular, a recent review reported that lower levels of ghrelin would favour thyroid cell proliferation, whereas supra-physiological levels would have an inhibitory effect *(Santini 2014)*.

Obesity-induced inflammation involves other inflammatory components that could contribute to tumorigenesis. These components include matrix metalloproteinases (MMPs), which are associated with cancer-cell invasion and metastasis *(Egeblad 2002, Marecko 2014, Wang 2013, Wang 2014)*.

Finally, the possible association between obesity and autoimmune thyroid diseases might also play a role because of the link between chronic autoimmune thyroiditis and thyroid cancer *(Cunha 2011)*.

Intrisic pathway

Genetic alterations

The intrinsic via is driven by the most frequent genetic alterations associated with DTC, such as RET/PTC rearrangement and BRAF point mutation. Up to 70% of PTCs express non-overlapping mutations of RET, TRKA, RAS and BRAF genes, which encode transcription of components of the mitogen-activated protein kinase (MAPK) cascade *(Guarino 2010)*. Both point-mutations and genetic rearrangements can promote the constitutive activation of the tyrosine kinase activity of RET in the absence of ligands. Activation of RET by physiological ligands or by oncogenic conversion results in the phosphorylation of intracellular tyrosine residues, which serve as docking sites for the recruitment of signalling adapters *(Takahashi 2001, Kimura 2003, Melillo 2016)*.

Local activities engaged in thyroid tumorigenesis and thyroid cancer progression are positively influenced by the activation of oncoprotein-mediated signalling present in epithelial cancer cells. Studies investigating the role of the RET/PTC3 oncoprotein in the recruitment of immune cell populations into the tumour site *(Powell 1998, Castellone 2004, Borrello 2005, Puxeddu 2005)* showed that the transplantation of RET/PTC3 expressing thyrocytes activates an inflammatory transcriptional program both in vitro and in vivo, and PTC-like lesions in mice were characterized by a leukocytic infiltrate mainly constituted by macrophages, with parallel increase in cytokine production within the tumour *(Russell 2004)*. Main humoral components of this program include mediators responsible for different pro-tumour effects, such as growth factors implicated in leucocyte recruitment and survival (G-CSF; GM-CSF, M-CSF), chemokines (CCL2, CXCL12), chemokines receptors (i.e. CXCR4) implicated in monocyte recruitment, angiogenesis and

tumour-cell homing to lymph nodes, IL-8, L-selectin and proteases responsible for tumour invasion and dissemination. RET/PTC3-positive thyroid cancer were also found to induce recruitment of CD11b+, Gr1+ cells capable of mediating tumour escape from the immune surveillance *(Pufnock 2009)*, whereas the expression of the RET/PTC3 isoform in a rat thyroid cell line (PC Cl3) was demonstrated to increase NF-kB DNA-binding activity with consequent increase in the pro-inflammatory cytokine secretion *(Russell 2003)*.

NF-kB ia a transcription factors laying at the intersection between the intrinsic and extrinsic proinflammatory pathways related to tumorigenesis *(Mantovani 2008)*, and high constitutive expression of NF-κB is a primary feature of cancer cells but not normal cells, indicating a crucial role for NF-κB in regulating tumorigenesis *(Karin 2006)*. NF-κB comprises a family of transcription factors involved in transcription of different genes controlling apoptosis, immune response and inflammation, as well as cancer development and progression. Activation of NF-κB results from different signalling pathways triggered by cytokines, growth factors, and tyrosine kinases *(Pacifico 2010, Gallel 2008)*. NF-κB is also recognized to play a major role in the initiation and progression of thyroid carcinoma *(Bonmarito 2011, Li 2012)*. In thyroid cancer cells, oncogenic proteins RET/PTC, RAS and BRAF can induce NF-κB activation in PTC, FTC, and MTC, while constitutive de-regulated NF-κB activity has been found in ATC *(Pacifico 2010)*. In a subset of PTCs associated with unfavourable outcome, it has been shown that NF-κB-mediated anti-apoptotic effects are enhanced by over-activation of Ras-related C3 botulinum toxin substrate 1 (RAC-1b), a hyperactive variant of the RAS superfamily of small GTP-binding proteins *(Faria 2017)*.

The immune network

Conflicting reports deal with the association between the prognosis of PTC and the degree of lymphocytic infiltration surrounding and/or inside the tumour *(Hirabayashi 1965, Clark 1980, Aguayo 1989, Matsubayashi 1995)*. Several studies suggest that the immune response might be important in preventing metastases and recurrence of thyroid cancer, improving disease-free survival *(Cunha 2011, Ehlers 2014)*. On the contrary, other studies showed that patients with tumor-associated lymphocytes exhibited higher disease stage and increased incidence of invasion and lymph node metastases compared to patients without lymphocytes, or with background thyroiditis *(French 2012, Quing 2012,*

Yu 2013). Moreover, recent studies *(Muzza 2010, Stassi 2003)* showed important clinical implication of autoimmunity on tumor behaviour; in particular, Stassi et al *(Stassi 2003)* demostrated that IL-4 and IL-10 activation induce thyroid cancer cells resistance to chemoterapeutic agents.

Autoimmunity stimulates the production of higher levels of proinflammatory cytokines with growth factor activity (IL-17, IFN-γ and TNF-α) and the angiogenesis enhanced by TNF-α and VEGF *(Ehlers 2014, Fajardo 1992, Ji 2012, Mardente 2010, Landskron 2014, Felicetti 2017)*; moreover, HMGB1 and nitric oxide, detected in both thyroiditis and PTC patients, promote matrix remodelling, inhibit immune response and are able to suppress cell cycle regulators, thus increasing the risk of PTC *(Mardente 2012)*. In addition, reactive oxygen species contribute to DNA damage and promote the epithelial-to-mesenchymal transition *(Wang 2014)*. Two hypotheses may explain the association between autoimmune thyroiditis and differentiated thyroid cancer. In both cases, the main actor is RET/PTC rearrangement, modulating the autoimmune response *(Ehlers 2014, Felicetti 2017, Borrello 2008)*. This rearrangement, considered specific for PTC, is also expressed in some non-neoplastic conditions such as Hashimoto's thyroiditis *(Wirtschafter 1997)*. In vivo studies showed that RET/PTC seems to be more represented in PTC associated with thyroiditis, whereas BRAFV600E in PTC alone *(Powell 1998, Muzza 2010)*. According to the first hypothesis, free radicals production, cytokine secretion, cellular proliferation as well as other phenomena related to local inflammation might predispose to the rearrangement in follicular cells *(Gandhi 2006)*. The second hypothesis is supported by the observation that RET/PTC3 rearrangement express high levels of proinflammatory cytokines and proteins involved in the immune response *(Melillo 2010, Puxeddu 2005, Rhoden 2006, Russell 2003)*. Furthermore, RET/PTC1 rearrangement is able to induce the expression of different genes involved in inflammation and tumor invasion, including chemokines, chemokine receptors, cytokines, adhesion molecules and matrix-degrading enzymes *(Borrello 2005)*. Other gene alterations have been proposed to explain the association between thyroid cancer and autoimmune thyroiditis. For example, p63 protein is commonly expressed in both PTC and Hashimoto thyroiditis *(Unger 2003)*, suggesting p63 expression as a potential link between these conditions *(Pellegrini 2001)*. Moreover, the increased expression of p-Akt, Akt1, and Akt2 in thyroid cancer and

autoimmune thyroiditis suggests PI3K/Akt pathway to be involved in both disorders *(Larson 2007)*.

Finally, recent analysis has focused on immune checkpoints as a prognostic and therapeutic tool for DTCs. In a study assessing immunostaining and mRNA levels of programmed death-ligand 1 (PD-L1), a macrophage-related cell surface glycoprotein regulating local inflammatory responses, more intense expression was observed in samples from DTCs than those from benign tumors, and increasing PD-L1 mRNA expression was demonstrated in more advanced tumor stages *(Cunha 2013)*. Similarly, an increased expression of PD-L1 has been observed in advanced DTCs and ATCs, both at the cellular level and on tumour-associated lymphocytes *(Bastman 2016)*. While these findings await confirmation in Tregs, TAMs, and immature dendritic cells, it appears feasible that studies focusing on immune checkpoint inhibitors in DTCs could lead to way to test new therapeutic strategies *(Cunha 2017)*.

Inflammation and MTC

Data regarding the potential link between MTC and cancer-related inflammation (CRI) originate from few in vitro studies.

In MTC, the intrinsic pathway, which is driven by genetic alterations associated with thyroid carcinogenesis, seems to play a major role in this link. Germline point mutations of proto-oncogene RET are known to be responsible for almost all familial MTC in multiple endocrine neoplasia (MEN) type 2A and 2B, and familial medullary thyroid carcinoma (FMTC), while somatic point mutations are found in up to 50% of patients with sporadic MTC *(Haugen 2016)*.

RET encodes for the tyrosine kinase receptor of growth factors belonging to the Glial cell-Derived Neurotrophic Factor (GDNF) family, the stimulation of which activates a variety of signalling pathways, such as the RAS/ERK, the PI3-K/AKT and the MAPK pathways, which are involved in cell survival and differentiation. Gain-of-function mutations of RET cause a constitutive activation of the tyrosine kinase activity of the receptor in the absence of ligands, leading to tumour development and progression *(Iwahashi 2002)*.

In vitro studies *(Iwahashi 2002)* demonstrated that GDNF stimulation induced high level of interleukin-8 (IL-8) production in TT medullary thyroid carcinoma cells. IL-8 is a pro-inflammatory, mitogenic and proangiogenic chemokine that is known to be involved directly in tumour growth, cell migration, and angiogenesis in an autocrine or paracrine way, or indirectly by attracting infiltration cells, including neutrophils and macrophages; therefore, its expression in tumour cells may affect their biological properties such as invasion and metastatic ability *(Iwahashi 2002, Liotti 2012)*.

Transcription of IL-8 is known to depend on activation of NF-IL-6 and NF-kB *(Iwahashi 2002, Mukaida 1994)*.

Two reports have associated RET-mediated carcinogenesis with NF-κB activation, so far *(Gallel 2008, Ludwig 2001)*. Ludwig et al. *(Ludwig 2001)* found that NF-κB was strongly expressed in tissue specimens from parafollicular C-cell carcinomas, and in vitro data suggested that NF-κB-dependent transcription plays an essential role in the development of MTC induced by both oncogenic RET isoforms, i.e. those harboring the mutations C634R or M918T, responsible for MEN 2A and MEN 2B, respectively. Gallel et al. *(Gallel 2008)* also demonstrated that the expression of mutated RET induces an increase in NF-κB DNA-binding activity and a consequent increase in pro-inflammatory cytokine secretion.

RET-mediated transformation would be dependent on NF-κB delivered anti-apoptotic and mitogenic signals. Since most part of pro-inflammatory molecules are under NF-κB transcriptional control, it has been hypothesized that NF-κB could be involved in the regulation of pro-inflammatory program of thyroid cells, and this event would contribute to the onset of thyroid cancer *(Pacifico 2010)*.

Inflammation and ATC

There are studies suggesting that inflammation could also be a key factor involved in the development of ATC, one of the most lethal human malignancies. Many characteristics of the inflammatory status leading to enhanced tumour growth, invasion, angiogenesis, and metastasis, are similar between ATC and PDTC, that is the bridge between DTC and ATC.

Compared to DTC and normal thyroid, ATC and PDTC show an increased amount of TAMs accounting for about more than 50% of immune cells infiltrating ATC. Infiltrate of TAMs is higher in PDTCs and ATCs than in PTCs and FTCs, and results positively correlated with the

poor prognosis of PDTCs *(Ryder 2008)*. TAMs usually form a "microglia-like" structure that is in close contact with cancer cells, and their inter-connection and density correlate with invasive features and worse prognosis of the tumor *(Caillou 2011, Visciano 2015, Galdiero 2016)*, as confirmed by the evidence that TAMs infiltrate promoted the invasiveness of ATC cell lines in vitro through production of CXCL8/IL-8 *(Galdiero 2016)*. In contrast to TAMs, infiltration of lymphocytes and dendritic cells, which are involved in antitumor response *(Cunha 2014, Poschke 2011)*, is reduced or absent in ATC *(Ugolini 2007)*. In ATC cell lines, CXCL8/IL-8 plays a central role in cell proliferation both during unstimulated conditions and under the effect of pro-inflammatory stimuli, such as IL-1 and TNF-α *(Galdiero 2016, Rotondi 2015)*. Recruitment of neutrophils within the thyroid gland, a crucial metastasis-promoting factor, is dependent on the amount of CXCL8 produced by the tumor cells when exposed to TNF-α (*Rotondi 2015, De Larco 2004)*. Moreover, there is evidence that induction of reduced expression of CXCL8/IL-8 and MCP-1/CCL2 pathways by the oncolytic adenovirus dl922-947 is able to impair angiogenesis and macrophage infiltration, as well as it promotes ATC cell death in vitro and tumor regression in vivo *(Passaro 2016)*. A recent study in a xenograft mouse model showed that cytokines expressed in ATC cell lines and tumour tissues, including IL-8, TGF-α, and TNF-α, were all down-regulated by suppression of ubiquitin-like containing PHD and RING finger domains 1 (UHRF1), involved in the DNA base excision repair (BER) pathway. This UHRF1-mediated effect was found to be associated with inhibited proliferation of ATC, both in vitro and in vivo *(Wang 2016)*.

Moreover, inflammatory conditions relating to production of INF-y and TNF-a can promote the autocrine production of IL4, IL10, CXCL1/GROa, CXCL10/IP-10 in primary human ATC cells, as well as in DTCs, and contribute to up-regulate anti-apoptotic pathways and chemo-resistance *(Stassi 2003, Rotondi 2013)*. Mast cells are also present in ATC and their infiltrate is directly correlated with tumour invasiveness through production of soluble factors involved in epithelial-to-mesenchymal transition, with CXCL8/IL-8 again acting as main effector of this mechanism *(Visciano 2015)*. Furthermore, mast cell-derived CXCL1/GRO-a and CXCL10/IP10 production increased ATC proliferation through the engagement of CXCR2 and CXCR3 expressed on thyroid cells *(Melillo 2010)*. Interestingly, the activation of the CXCL10-CXCR3 axis was also induced by NK cell

migration in ATC cell lines. Prostaglandin-E2 was identified as the main responsible for the ATC-mediated NK cell suppression *(Wennerberg 2014)*.

At odds with results obtained in DTCs, there is no evidence of a connection between the oncogenetic background of ATCs and inflammation. Likewise, no associations have been found between Hashimoto's thyroiditis or thyroid lymphoma and ATC incidence.

It is worth noting that observational studies in humans have investigated different inflammatory biomarkers as a tool to assess aggressiveness of different thyroid cancers subtypes. The neutrophil-to-lymphocyte ratio, a simple surrogate index of the systemic inflammatory response, was shown to be a prognostic factor in some types of cancers. In a cohort of 3,870 patients affected by benign or malignant thyroid diseases, the neutrophil-to-lymphocyte ratio differed between tumour cancer subtypes and was 3.8.-fold higher in ATC than in PDTC or PTC patients *(Cho 2015)*. Moreover, also eosinophilia refractory to steroids has been recently reported in an ATC patient *(Shiraishi 2015)*. Finally, serum levels of IL10 and C-reactive protein in ATC patients were directly correlated with higher peripheral blood myeloid cells (MDSSCs), which are known to be immunosuppressive and cancer promoting *(Suzuki 2013)*.

Conclusions

The link between inflammation and thyroid cancer involves multiple elements of the immune system, ECM, stroma, and AT. The role of these link is complex: the pro-tumoral activity of inflammation is opposed to anti-tumor functions favoring protection against cancer progression. This paradox may be explained by the specific circuits expressed within the tumour microenvironment and by the abundance and activation state of different cell types in the tumour site.

Within the tumour microenvironment, inflammatory cells, belonging both to innate (macrophages) and adaptive (lymphocytes) immune responses, are interconnected with fibroblasts, endothelial cells, adipocytes, and ECM through cytokines, chemokines and adipocytokines.

Cancer-related inflammation could represent an important target for innovative diagnostic and therapeutic strategies in thyroid cancer. The molecular patterns of cytokines and chemokines are key orchestrators and could explain the involvement of the

immune system in tumour progression. In fact, anticancer immunotherapy, in particular the immune checkpoint inhibitors, act promoting lymphocyte activation in order to destroy cancer cells and counteract immune-suppressive signals produced by cancer cells. By doing this, they also activate immune memory, leading to a sustained anti-tumour response.

Further informations on the inflammatory microenvironment may help to explain tumour aggressive behaviour and identify potential new targets of therapy.

Circulating adipokines and metabolic setting in differentiated thyroid cancer: an observational study

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Abstract

Background. The associative link relating insulin resistance (IR) and adipokines to the occurrence and phenotype of differentiated thyroid cancer (DTC) is unknown. Aim of this study was to evaluate the relationship between insulin resistance (IR) and adipokines in DTC patients, as compared with carriers of benign thyroid diseases (BTD) and controls free of thyroid diseases.

Methods. This observational study enrolled 77 subjects phenotyped as DTC (N=30), BTD (N=27) and healthy subjects (N=20). Each subject underwent analysis of anthropometric parameters, thyroid function and autoimmunity, insulin resistance (HOMA-IR) and levels of unacylated (UAG) and acylated ghrelin (AG), obestatin, leptin and adiponectin . Multivariate regression models were used to test the predictive role of metabolic correlates on thyroid phenotypes and DTC extension.

Results. The three groups showed similar age, gender distribution, smoking habit, BMI and thyroid parameters. Obestatin was significantly higher in DTC group compared to BTD ($p<0.05$) and control subjects ($p<0.0001$). DTC and BTD groups showed higher levels of UAG ($p<0.01$ for both) and AG ($p<0.05$ for both). Leptin levels were comparable between groups, whereas adiponectin levels were lower in DTC group compared to BTD group ($p<0.0001$) and controls ($p<0.01$). In parallel, HOMA-IR was higher in DTC than BTS ($p<0.05$) and control group ($p<0.01$). Stepwise multivariable regression analysis showed that obestatin and UAG levels were independent predictor of DTC ($p=0.01$ for both). In an analysis restricted to the DTC group, multinomial regression analysis showed that obestatin levels were associated with the absence of lymph node metastases ($p<0.05$).

Conclusion. Our results highlight a potential association between metabolic setting, circulating adipokines, and thyroid tumorigenesis . Further studies are needed to validate these findings.

Background

The incidence of differentiated thyroid cancer (DTC) has been rising in many countries over the last few decades *(Pazaitou-Panayiotou 2013, Pagano 2017)* along with a parallel increase in obesity and metabolic diseases *(Pazaitou-Panayiotou 2013)*.

Even if the rising incidence of DTC could be attributed to an improved diagnostic accuracy, which results in "over-diagnosis" of microcarcinomas *(Pazaitou-Panayiotou 2013a, Elisei 2010, Lee 2012, Grodski 2008)*, an increasing prevalence of more aggressive forms of DTCs suggests the real existence of a changing pattern of incidence *(Gomez Segovia 2004, Enewold 2009, Pazaitou-Panayiotou 2013b)*. Environmental and lifestyle factors, such as obesity and insulin resistance (IR), seem to be implicated in the pathogenesis of thyroid cancer *(Pagano 2017, Marcello 2014, Santini 2014)*. Although the pathways linking obesity or IR to thyroid cancer remain largely unknown, a potential role for insulin, growth hormone (GH) secretagogues, and adipokines has been postulated *(Santini 2014)*. The insulin-cancer hypothesis assumes that hyperinsulinemia could decrease the concentrations of IGF-binding protein 1 (IGFBP1) and IGFBP2, which would increase the bioavailable free IGF-1 levels *(Almquist 2011)*. IGF-1 has mitogenic and anti-apoptotic effects, which favour tumorigenesis *(Santini 2014)*. Other factors potentially linking obesity, IR and DTC could be represented by ghrelin, a GH secretagogue, and obestatin, which are expressed in tumor cancer tissues albeit with inhomogeneous patterns *(Karaoglu 2009)*. There are studies suggesting that the adipocytokine network could play a role in thyroid tumorigenesis. Leptin is an adipokine predominantly secreted by the adipose tissue *(Piya 2013)* that is involved in the regulation of food intake and resting energy expenditure, as well as activation of monocytes and macrophages, and stimulation of angiogenesis and cell proliferation *(Kwon 2013)*. Several studies investigated the effect of leptin on thyroid cancer cells *(Santini 2014)*. Leptin was shown to promote cell migration in PTC cells *(Cheng 2010)*, to stimulate a more aggressive cancer phenotype *(Uddin 2010)* and to promote the de-differentiation of thyroid cancer cells *(Kim 2013)*. Adiponectin is an adipokine with anti-inflammatory properties produced by adipocytes. It is able to improve insulin sensitivity, influence cell proliferation, and regulate the balance of anti- and pro-inflammatory molecules and cells *(Marcello 2014, Li 2009)*. Serum adiponectin levels are inversely correlated with the presence of DTC

(Mitsiades 2011), and the absence of adiponectin receptors has been shown to be associated with extrathyroidal extension, multicentricity and higher TNM stage of DTCs *(Cheng 2013)*.

To date, the causal association relating IR and adipokines to the presence and phenotype of DTC is unknown and the associative studies so far published have presented discordant data. Therefore, the aim of our observational study was to evaluate the relationship between IR and adipokines in DTC patients, as compared with carriers of benign thyroid diseases and subjects without thyroid diseases.

Methods

Patients

The study enrolled 77 subjects, consisting of 57 adults undergoing total thyroidectomy because of benign thyroid disease (uninodular or multinodular goitre, follicular adenoma) or suspected DTC on the basis of cytological examination according to Bethesda criteria *(Cibas 2017)* and classified as TIR3B, TIR4, TIR5. Twenty healthy subjects without thyroid disease served as control group.

Following blood sampling for the purpose of the study, patients who underwent thyroidectomy were followingly divided into two groups, according to the post-surgical histological diagnosis:

- DTC group: 30 patients diagnosed with papillary or follicular thyroid cancer
- BTD Group: 27 patients diagnosed with benign thyroid diseases.

Subjects with poorly differentiated, anaplastic, medullary thyroid carcinomas, secondary tumors, history of medical neck irradiation, hormone therapies and treatments interfering with insulin sensitivity were excluded from the analysis.

The experimental procedure was approved by the ad hoc Ethical Research Committee of Novara, Italy. A written informed consent was obtained from all patients and controls. The study protocol was conformed to the guidelines of the European Convention on Human Rights and Biomedicine concerning biomedical research.

Body measurements

All subjects underwent body measurements wearing light underwear, in fasting conditions after voiding. Weight and height were measured in the nearest 0.1 kg and 0.1 cm, respectively, using standard methods. BMI was expressed as body mass (kg)/height $(m)^2$. Overweight was defined for any BMI between 25 and 29.9 kg/m^2 and obesity for any BMI over 30 kg/m^2. Waist circumference (WC) was measured midway between the lowest rib and the top of the iliac crest after gentle expiration.

Laboratory tests

Blood samples were drawn under fasting conditions, centrifugated, and stored at -20°C or -80°C, until assay.

Undiluited serum samples were assayed for thyroid stimulating hormone (TSH), free-tyroxine (fT4) and free-triiodothyronine (fT3) using an automated chemiluminescence assay system (ADVIA Centaur Systems TSH3/fT4/fT3 Ultra Ready Pack, Siemens Healthcare Diagnostics, Milan).

Serum levels of thyroglobulin (Tg) were determined using an automated chemiluminescence method (LIAISON XL, DiaSorin S.p.A, Saluggia).

Plasmatic levels of anti-Tg and anti-thyroperoxidase (TPO) antibodies were assessed using an automated chemiluminescence assay system (Anti-Tg, Anti-TPO Ready Pack, Siemens Healthcare Diagnostics, Milan).

Plasma glucose levels were determined using an enzymatic method (ADVIA 1800 Chemistry System, Siemens Healthineers, Milan). Serum insulin levels were obtained using direct chemiluminescence method (ADVIA Centaur IRI, Siemens, Milan). Insulin resistance was calculated by the homeostatic model of insulin resistance (HOMA-IR) index: insulin (mIU/mL) x [glucose (mmol/L)/22.5] *(Matthews 1985)*. A HOMA-IR value greater than 2.5 was considered indicative of insulin resistance *(Muniyappa 2008)*.

For hormone assays, assay procedure was performed in accordance with the manufacturer's instruction and the samples were analyzed in duplicate.

Serum leptin levels were assessed using a commercially available human ELISA kit (Mediagnost, Reutlinger, Germany). Intra-assay CV and inter-assay CV of leptin were less than 10%. Minimum detectable concentration was 0.2 ng/mL.

Serum adiponectin levels were determined using a commercially available human ELISA kit (Mediagnost). Intra-assay CV was less than 6.7% and inter-assay CV was less than 4.7%. Minimum detectable concentration was 0.6 ng/mL.

Serum obestatin concentrations were assessed using a commercially available human EIA kit (Yanaihara Institute Inc, Awakura, Japan). Intra-assay CV was 3.5-9.9% and inter-assay CV was 5.6-9.0%. Minimum detectable concentration was 0.231 ng/mL.

Plasma unacylated ghrelin (UAG) levels were determined using a commercially available human ELISA kit (BioVendor Research and Diagnostic Product, Czech Republic). Intra-assay CV was 3.2-11.8% and inter-assay CV was 3.8-13.2%. Minimum detectable concentration was 6 pg/mL.

Plasma acylated ghrelin (AG) levels were assessed using a commercially available human ELISA kit (BioVendor). Intra-assay CV was 2.9-11.8% and inter-assay CV was 3.4-14.4%. Minimum detectable concentration was 5 pg/mL.

Thyroid cytology and histology

The cytology specimens were evaluated and classified according to the international guidelines *(Haugen 2016, Nardi 2014)*. Histological slides were reviewed by two independent pathologists for the purpose of this study. For all cases, tumor-associated thyroiditis was assessed. The tumor size, number of foci, focality, extension, presence of loco-regional and/or distant metastases were also reported and classified according to the 2010 tumor-node-metastases (TNM) system *(Edge 2010)*.

Neck ultrasound (US)

Pre-surgical US was routinely performed in all patients. The study was conducted using a "My Lab 25 Gold" (ESAOTE S.p.A, Genova, Italy) equipped with a linear transducer of 7.5 MHz. Sonographic features predictive of malignancy were considered according to

American Thyroid Association and AACE-AME guidelines *(Haugen 2016, Gharib 2016)*. Cervical lymphadenopathies and their US features were also evaluated.

Data analysis

Statistical analysis was performed using SPSS version 21 (Somers, NY, USA) on log transformed data to correct for the non-Gaussian distribution obtained by the Shapiro–Wilk test. Values were expressed as median and interquartile ranges (IQ). For comparative analysis, ANOVA between the 3 groups was used. Spearman's correlation analysis was used to identify significant associations between variables of interest. ANCOVA multinomial regression analysis was used to evaluate the association of adipokines levels with histological characteristics of DTCs. Stepwise multivariate regression analysis was used to evaluate the independent association of metabolic, anthropometric or biochemical parameters with adipokines and HOMA-IR. β coefficients and related significance values obtained from the models were reported. $P<0.05$ was considered as statistically significant.

Results

Analysis on all study groups

The anthropometric and biochemical data of our population are reported in Table 1 (pag. 189).

Anthropometric parameters were comparable between the groups. Obesity (BMI > 30 kg/m2) was present in 20.8% of cases (6 patients with DTC, 8 patients with BTD and 2 controls) and overweight in 27.3% (7 patients with DTC, 6 patients with BTD and 8 controls). Before surgery, hypothyroidism under replacement therapy was present in 14% of patients (6 patients with DTC and 2 patients with BTD), while hyperthyroidism under methimazole treatment was present in 5.3% (3 patients with BTD).

Thyroid function parameters were comparable between the groups, whereas TPOAb levels were higher in DTC and BTD group with respect to the control group ($p<0.05$).

Compared to controls, we observed higher levels of UAG ($p<0.01$ for both) and AG ($p<0.05$ for both) in DTC and BTD groups. Obestatin concentrations were higher in DTC group as compared both to BTD group ($p<0.05$) and controls ($p<0.0001$), as well as in the BTD group with respect to controls ($p<0.0001$). Leptin levels were comparable between the 3 groups, whereas adiponectin levels were lower in DTC group compared to BTD group ($p<0.0001$) and controls ($p<0.01$), and in BTD group with respect to controls ($p<0.0001$).

Analysis of metabolic parameters showed the occurrence of insulin resistance in 32.5% of the entire dataset (14 patients with DTC, 8 patients with BTD and 3 controls). The DTC group showed higher insulin levels and HOMA-IR than BTD (Insulin: $p<0.01$, HOMA-IR: $p<0.05$) and control group (Insulin: $p<0.01$, HOMA-IR: $p<0.01$).

In bivariate correlation analysis across the three groups (Table 2, pag. 190), expected positive associations were documented between metabolic parameters, HOMA-IR and leptin levels. Both leptin and adiponectin were positively correlated with anti-TG antibodies, while adiponectin was also correlated with AbTPO levels. However the above mentioned correlations were lost after controlling for age, gender, BMI and thyroid phenotypes.

Stepwise multivariable regression analysis with a model weighted for gender, age, BMI and HOMA-IR documented that the presence of DTC (DTC=0, BTD=1, Controls=2) was independently predicted by obestatin and UAG levels (respectively $\beta=-0.339$ and $\beta=-0.357$, $p=0.01$).

Analysis of patients with DTC

Histopathological characteristics of all patients with DTC are summarized in Table 3 (pag. 191). As expected, the most frequent histotype was PTC (83.3%) and the most widespread variant was the classical one (36.0%), followed by other more aggressive variants such as tall cell, oncocytic, solid and Warthin-like variants. Capsular invasion was present in more than 40.0% of cases, bilaterality and multifocality respectively in 16.7% and 26.7% of patients. Overall, 60.0% of tumours were classified as stage I, followed by stage IV (16.7%), with high frequency of thyroid capsular invasion (43.3%). Tumors were bilateral in 16.7% and multifocal in the 26.7% of patients. Among patients who

underwent lymphadenectomy, 38.9% had lymph node metastases. Finally, tumour-associated thyroiditis was histologically found in 23.3% of cases.

Bivariate correlation analysis restricted to the DTC group is reported in Table 4 (pag. 192). After adjusting for age, BMI and gender, only the negative association between obestatin levels and the presence of lymph node metastases ($r=-0.862$, $p<0.05$) and the positive association between obestatin and TPOAb titer ($r=0.856$, $p<0.05$) remained significant.

Multinomial logistic regression analysis, performed to study the association between adipokines levels or HOMA-IR and DTC histological characteristics, showed that obestatin levels were significantly associated with the absence of lymph node metastases (Odds Ratio (OR)=4.370, CI 95% 1.080-10.964, $p<0.05$).

Discussion

The present study analysed the relationship between IR, adipokines and DTC tumorigenesis and histopathological characteristics. Our results show that the prevalence of insulin resistance was higher in patients with DTC and that these subjects harbour higher level of obestatin than patient with benign nodules and controls. However in the analysis restricted to DTC cases, obestatin levels were inversely associated with the metastatic capacity of tumor. While leptin levels were comparable between groups, adiponectin levels are lower in patients with DTC in respect to subjects with benign nodules and controls. Moreover, in patients with thyroid nodules, either benign or malignant, we observed higher concentrations of UAG and AG compared to controls. Higher obestatin and UAG levels also represented predictors of the presence of malignancy in patients with thyroid nodules.

In the past few decades, the incidence of DTC has shown a steady increase worldwide *(Pagano 2017, Enewold 2009)*. Some evidence suggests that environmental and lifestyle factors can play an important role *(Pagano 2017, Leenhardt 2011)*. Among the potential risk factors involved in the changing epidemiology of TC, particular attention has been drawn to insulin resistance and metabolic syndrome, which have been also rapidly increasing worldwide due to widespread dietary and lifestyle changes *(Malaguarnera 2017)*. On this basis, it has been suggested that insulin resistance is associated with the

rising incidence of differentiated thyroid cancers. A small cross-sectional study detected an increased prevalence of insulin resistance in patients with DTC *(Rezzonico 2009)*, and a recent study of Bae et al. *(Bae 2016)* showed a clearcut association relating insulin and HOMA-IR index to the risk of PTC and the multifocality of this disease. Our results confirmed these findings. In fact, we found that patients with DTC harbour higher insulin levels and HOMA-IR index with respect to patients with benign nodules and subjects without thyroid diseases. Insulin resistance is known to be an important risk factor responsible for carcinogenesis *(Inoue 2012)*. Insulin shares structural homology with IGF-1, which binds to the IGF-1 receptor and functions as a potent growth factor with key roles in malignant transformation, tumor progression, and metastasis in various kinds of cell types *(Clemmons 1989, Frasca 2008)*. It has been shown that IGF-1, its receptor, and insulin receptors are expressed in thyroid cancers *(Vella 2001)*, suggesting that hyperinsulinemia, a consequence of insulin resistance, may play a role in thyroid carcinogenesis. However, we failed to demonstrate direct associations between insulin levels and DTC, suggesting that the metabolic setting rather than insulin levels could play a role in this relationship.This circumstance would agree with the evidence that insulin resistance enhances the proinflammatory state by decreasing the production of anti-inflammatory adipokines, such as adiponectin, and increasing the production of pro-inflammatory adipokines, such as leptin, which could interact with molecular pathways involved in tumor development *(Catalan 2013)*.

Adipokines are a subset of cytokines produced by the adipose tissue *(Cunha 2014)*. They are involved not only in immune responses, but also in the regulation of appetite and energy balance, insulin sensitivity, angiogenesis, blood pressure regulation, and lipid metabolism *(Kwon 2013)*. In particular, leptin is involved in the control of food intake through satiety sensation, regulation of energy expenditure, the activation of monocytes and macrophages, stimulation of VEGF, angiogenesis, cell proliferation, and the suppression of anti-inflammatory cytokines. The induction of leptin responses and effects involves its binding to leptin receptor b (ObR), leading to the activation of intracellular signals through JAK2, STAT3, and AMPK *(Kwon 2013)*. These molecules will then regulate several pathways of pivotal importance to cancers, such as the AKT/mTOR/PI3K and ERK/MAPK pathways, involved in cell proliferation and survival *(Surmacz 2013)*. A relationship of this molecule with DTC and other cancers has demonstrated *(Dutta 2012,*

Vansaun 2013). As regards DTC, leptin was found to be associated with a high risk of lymph node metastases *(Cheng 2010)* and it seems to be involved in the clinical phenotype of the tumours as well as in the migration of thyroid cell, promoting metastases formation and diffusion *(Cheng 2010, Kim 2013, Cheng 2011, Cheng 2012, Zhang 2013)*. Although in our study failed to detect differences in serum leptin levels across the three groups, we observed a significant association between circulating leptin and the presence of lymph node metastases, which was lost after controlling for confounders (age, BMI and gender). However we have to consider that the serum concentration of leptin may not reflect its action on thyroid tissue. Furthermore, the higher levels of this adipokine found in subjects with benign nodules, would require future analysis to evaluate its modulatory role in the hyperplastic phase of the thyroid follicle. Subsequently, in the process of neoplastic transformation of the thyroid follicle, the loss of leptin expression could positively regulate the process of oncogenic transformation with unknown mechanisms, similarly to what happens in the ovary cancer *(Jin 2016)*.

Adiponectin is an adipokine with strong anti-inflammatory properties. It is able to improve insulin sensitivity, influence cell proliferation, and regulate the balance of anti- and pro-inflammatory molecules and cells *(Yokota 2000, Kumada 2004, Takemura 2007)*. Owing to its complex anti-proliferative and inflammation-restraining functions, adiponectin has been suggested to have anti-neoplastic properties in some types of cancers *(Marcello 2014)*. Our results showed lower adiponectin levels in patients with thyroid cancer. Similar findings were shown by Mitsiades et al. *(Mitsiades 2011)*, who demonstrated that serum adiponectin levels are inversely associated with the prevalence of DTCs, suggesting a potential protective effect of adiponectin against the development of this cancer. The mechanisms by which adiponectin may act on thyroid cancer still remain to be identified but it may include a protection against the development of insulin resistance *(Yadav 2013)*, in particular through the activation of the adenosine monophosphate kinase (AMPK) pathway *(Cheng 2013, Yamauchi 2002, Zakikhani 2008)*. Adiponectin has also been shown to directly inhibit angiogenesis and promote apoptosis in vivo, through the activation of the caspase cascade *(Brakenhielm 2004)*. Low adiponectin has been proposed as a possible mediator in the association between BMI and cancer risk *(Dalamaga 2012)*.

Ghrelin is implicated in several processes of cancer progression including cell proliferation, cell migration and invasion, angiogenesis, and apoptosis, probably via an autocrine/paracrine mechanism *(Ucan 2017)*. Some reports demonstrated that ghrelin may have an inhibitory effect in the proliferation of some cancer types, including thyroid, prostate, breast and small cell lung carcinoma *(Chopin 2011)*. However, the exact role of ghrelin in thyroid tumorigenesis is still debated. Ucan and colleagues *(Ucan 2017)* did not found any significant differences in serum ghrelin levels between patients with PTC and healthy controls. Our study demonstrated that AG concentration were higher in the patients with malignant and benign thyroid nodules with respect to controls. These findings seem to confirm the hypothesized role of AG in promoting tumorigenesis *(Malendowicz 2009, Markowska 2009)*. In particular, the increased expression of this molecule could influence thyroid tumor proliferation through deregulation of GH and IGF-1 secretion, factors involved in cancer growth *(Samani 2007)*.

With regard to UAG, our data showed that this hormone was higher in patients with DTC and benign thyroid nodules. Moreover, we found that the presence of DTC was independently predicted by UAG. Recently, UAG has been hypothesized to be involved in various biological activities, including the inhibitory role on AG *(Delhanty 2013)*, the anti-proliferative and pro-apoptotic capacity on different cell lines in vitro *(Cassoni 2006)*. However, these are preliminary results and its role is still debated. Further prospective studies investigating ghrelin expression in DTCs and its association with serum ghrelin levels could be helpful to clarify this issue.

Finally, the role of obestatin in promoting thyroid cancer tumorigenesis is still controversial. However, this molecule appears very interesting for its probable involvement in cell proliferation through AKT-dependent signalling *(Chopin 2011, Camina 2007, Pazos 2007).* In previous studies, Volante et al. *(Volante 2009)* found obestatin expression in medullary, papillary, follicular and poorly differentiated thyroid cancer. The authors detected obestatin immunoreactivity mostly in ghrelin-positive areas of DTC, whereas there was no obestatin expression in normal thyroid tissue *(Volante 2009)*. On the contrary, Karaoglu et al. demonstrated obestatin expression in healthy thyroid, Hashimoto thyroiditis and PTC without evident differences in the intensity of reaction *(Karaoglu 2009)*. In a recent study, obestatin immunoreactivity was observed in benign nodules, as well as cancer cells *(Gurgul 2015)*. However, the intensity of

immunohistochemical expression was poor and obestatin-positive cells were accompanied by regions without any immunoreactivity *(Volante 2009)*. In our study, serum obestatin concentrations were, overall, higher in patients with DTC with respect to subjects with BTD and controls, thus representing a predictor of the presence of DTC. Interestingly, within the DTC group obestatin levels were significantly associated with the absence of lymph node metastases. Together, our data suggest a dual role for obestatin, such that its circulating levels could reflect thyroid tumorigenesis and represent a biomarker of the metabolic setting of DTC, but at the same time this adipokine could reflect lower proneness to the metastatic diffusion of DTC.

Conclusions

In conclusion, the role of these metabolic biomarkers in thyroid tumorigenesis appears very interesting due to their potential involvement in cell proliferation. We speculate that the expression of these factors may be altered in different phases of thyroid cell proliferation, and could reflect the influence of these markers on the pathogenesis of nodular goitre and thyroid cancer. Furthermore, since the thyroid gland is associated with the maintenance of energy balance, the relationship between IR, adipokines network and thyroid tumorigenesis is worthy of consideration.

With the complexity of insulin resistance-related pathways still awaiting full clarification in DTC patients, an array of metabolic molecules could represent novel therapeutic targets in thyroid health and disease.

Tables

Table 1. Anthropometric and biochemical data obtained in the 2 groups and controls**.**

Parameters [Median (IQ)]	Controls	BTD Group	DTC Group
GENDER **N. males** (%) **N. females** (%)	 5 (25.0%) 15 (75.0%)	 4 (14.8%) 23 (85.2%)	 9 (30.0%) 21 (70.0%)
AGE (years)	47.0 (37.0-62.5)	56.0 (53.5-65.0)	50.0 (41.0-58.8)
SMOKE **N. No (%)** **N. Yes (%)** **N. Ex (%)**	 24 (70.0%) 3 (15.0%) 3 (15.0%)	 17 (63.0%) 5 (18.5%) 5 (18.5%)	 18 (60.0%) 4 (13.3%) 8 (26.7%)
WEIGHT (Kg)	69.5 (58.0-73.3)	62.0 (58.0-80.5)	68.0 (57.5-83.8)
BMI (kg/m^2)	25.1 (22.7-26.4)	25.8 (21.9-30.3)	24.2 (22.1-28.2)
WC (cm)	86.5 (78.8-90.0)	90.0 (76.0-99.5)	93.0 (80.0-102.8)
TSH (µIU/mL)	1.6 (1.2-2.1)	1.3 (1.0-2.0)	1.5 (0.9-2.8)
fT4 (ng/dL)	1.2 (1.1-1.3)	1.1 (1.0-1.3)	1.1 (1.0-1.3)
fT3 (pg/mL)	3.1 (3.0-3.3)	3.4 (2.8-3.5)	3.1 (2.9-3.4)
TG (ng/dL)	17.2 (9.4-30.5)	43.3 (15.7-220.9) ^{§1}	11.9 (3.6-24.7) §
TGAb (IU/mL)	34.5 (21.3-52.0)	32.2 (15.0-54.5)	20.8 (13.5-57.5)
TPOAb (IU/mL)	33.0 (21.0-49.5)	41.2 (32.9-134.9) §	37.3 (13.5-81.5) §
GLUCOSE (mg/dL)	86.0 (83.5-94.5)	90.0 (81.5-98.0)	89.0 (80.8-97.8)
INSULIN (mIU/mL)	8.1 (6.5-10.1)	7.5 (5.8-11.5)	11.3 (8.4-18.5) ^{§1,*1}
HOMA-IR	1.6 (1.4-2-2)	1.6 (1.3-2.6)	2.5 (1.8-4.1) ^{§1,*}
UAG (pg/mL)	21.7 (11.8-30.8)	79.2 (39.0-129.8) ^{§1}	57.8 (28.9-101.2) ^{§1}
AG (pg/mL)	4.3 (2.4-6.2)	9.0 (5.3-16.5) §	5.7 (2.1-14.4) §
OBESTATIN (ng/mL)	2.5 (2.2-2.6)	4.6 (4.2-5.0) ^{§2}	5.0 (4.4-6.1) ^{§2,*}
LEPTIN (ng/mL)	10.6 (5.7-25.6)	16.8 (13.2-23.1)	11.6 (4.1-19.1)
ADIPONECTIN (ng/mL)	31.4 (15.2-41.0)	22.2 (17.7-31.1)	7.3 (4.5-17.7) ^{§1,*2}

Data are expressed as median (with interquartile range in parentheses) Comparison between populations was performed by ANOVA test and χ2 test. Significant differences are shown in bold characters.
Significant differences between controls and BTD or DTC Group: §p<0.05, ^{§1}p<0.01, ^{§2}p<0.0001
Significant differences between BTD and DTC group: *p<0.05, ^{*1}p<0.01, ^{*2}p<0.0001
BMI, Body Mass Index; WC, Waist Circumference; TSH, Thyroid Stimulating Hormone; fT4, free Thyroxine; fT3, free Triiodothyronine; TG, Thyroglobulin; TGAb, Anti-Thyroglobulin antibodies; TPOAb, anti-thyroperoxidase antibodies; HOMA-IR, Homeostatic Model of Insulin Resistance; UAG, unacylated ghrelin; AG, acylated ghrelin

Table 2. Spearman's correlation analysis between adipokines levels, HOMA-IR and anthropometric-biochemical parameters in the population as a whole. [1]p<0.05, [2]p<0.01, [3]p<0.001, [4]p<0.0001

Variables (Correlation coefficient)	UAG	AG	Obestatin	Leptin	Adiponectin	HOMA-IR
Gender	0.069	0.255	0.056	**0.312**[2]	-0.042	-0.150
Age	-0.154	-0.125	-0.151	**0.363**[2]	0.194	**0.273**[1]
BMI (kg/m^2)	-0.160	-0.079	-0.098	**0.606**[4]	-0.026	**0.561**[4]
WC (cm)	-0.037	-0.024	-0.140	**0.585**[4]	-0.154	**0.598**[4]
Glucose (mg/dL)	-0.017	-0.075	0.079	**0.304**[2]	0.210	**0.509**[4]
Insulin (mIU/mL)	-0.236	-0.213	-0.178	**0.453**[4]	**-0,279**[1]	**0.953**[4]
HOMA-IR	-0.202	-0.208	-0.138	**0.453**[4]	-0.195	-
TSH (µIU/mL)	-0.008	-0.090	0.087	-0.014	-0.049	0.068
fT4 (ng/dL)	0.101	0.138	-0.125	0.051	0.083	-0.007
fT3 (pg/mL)	-0.161	-0.099	0.007	0.006	0.028	-0.067
TG (ng/dL)	-0.126	-0,080	-0.022	-0.045	0.234	-0.191
TgAb (IU/mL)	0.032	0.045	-0.031	**0.263**[1]	**0.374**[2]	0.182
TPOAb (IU/mL)	0.258	0.241	0.072	0.161	**0.289**[1]	0.043

BMI, Body Mass Index; WC, Waist Circumference; HOMA-IR, Homeostatic Model of Insulin Resistance; TSH, Thyroid Stimulating Hormone; fT4, free Thyroxine; fT3, free Triiodothyronine; TG, Thyroglobulin; TGAb, Anti-Thyroglobulin antibodies; TPOAb, anti-thyroperoxidase antibodies

Table 3. Histopathological characteristics of all patients with DTC.

		N. of patients (%) (30 cases)
Cytology	Not performed	1 (3.3%)
	Thy 2	1 (3.3%)
	Thy 3f	10 (33.4%)
	Thy 4	5 (16.7%)
	Thy 5	13 (43.3%)
Histotype	PTC	25 (83.3%)
	FTC	5 (16.7%)
PTC variant	Classical	9 (36.0%)
	Follicular	5 (20.0%)
	Classical and Follicular	5 (20.0%)
	Other	6 (24.0%)
FTC variant	Minimally invasive	4 (80.0%)
	Invasive	1 (20.0%)
Tumour size (cm)	≤ 1 cm	6 (20.0%)
	1.0-2.0 cm	11 (36.7%)
	2.0-4.0 cm	11 (36.7%)
	>4 cm	2 (6.6%)
Multifocality	Present	8 (26.7%)
Bilaterality	Present	5 (16.7%)
Thyroid capsular invasion	Present	13 (43.3%)
Staging (TNM 2010)	Stage I	18 (60.0%)
	Stage II	4 (13.3%)
	Stage III	3 (10.0%)
	Stage IV	5 (16.7%)
Histological associated thyroiditis	Present	7 (23.3%)
Lymph node metastases	Present (18 lymphadenectomy)	7 (38.9%)

PTC, Papillary Thyroid Cancer; FTC, Follicular Thyroid Cancer

Table 4. Spearman's correlation analysis between adipokines levels and tumour characteristics and thyroid biochemical parameters in patients with DTC. [1]p<0.05, [2]p<0.01

Variables (Correlation coefficient)	UAG	AG	Obestatin	Leptin	Adiponectin	HOMA-IR
Tumour size	0.110	-0.004	**0.497**[1]	-0.250	0.303	-0.149
Histotype[*]	-0.028	0.000	-0.094	-0.175	0.323	-0.109
PTC variants[§]	0.156	0.101	-0.079	-0.011	0.306	-0.142
Bilaterality	-0.254	-0.085	0.158	0.316	-0.022	0.164
Multifocality	-0.089	0.022	0.227	0.194	0.231	0.074
Thyroid capsular invasion	-0.333	-0.210	0.136	0.207	-0.091	0.124
Lymph node metastases	-0.056	-0.280	**-0.779**[2]	**-0.439**[1]	0.187	0.073
Histological associated thyroiditis	-0.292	-0.280	-0.227	**0.501**[2]	0.106	0.308
Staging	-0.314	-0.340	0.272	-0.044	0.180	0.223
TSH	-0.222	-0.105	0.193	0.090	-0.120	0.111
fT4	0.197	0.128	-0.159	0.145	-0.084	-0.168
fT3	-0.189	-0.104	-0.095	-0.213	-0.151	-0.275
Tg	-0.089	-0.230	0.210	-0.229	0.043	-0.046
TgAB	0.017	0.007	-0.202	**0.443**[1]	0.281	0.307
TPOAb	0.279	0.300	-0.256	0.333	0.250	-0.082

[*]*PTC=0, FTC=1;* [§]*Classical=0, Follicular=1, Classical and Follicular=2, Other variants=3*

PTC, Papillary Thyroid Cancer; FTC, Follicular Thyroid Cancer; TSH, Thyroid Stimulating Hormone; fT4, free Thyroxine; fT3, free Triiodothyronine; Tg, Thyroglobulin; TGAb, Anti-Thyroglobulin antibodies; TPOAb, anti-thyroperoxidase antibodies

CHAPTER 3

The crosstalk between obesity and thyroid gland

Introduction

Body fat excess related to obesity results from the interaction between environmental and genetic factors, and a positive energy balance. This condition leads to adipocyte hypertrophy with resultant dysfunction of white adipose tissue, resulting in the development of hypoxia, oxidative stress, and inflammation *(Gregor 2011, Rupérez 2014)*. Due to the endocrine role of adipose tissue and its importance in homeostasis, the dysfunction of this tissue in obesity contributes to metabolic alterations in various organs and systems *(Bays 2008, Trayhurn 2013)*. In this respect, endocrine disorders stand out, and changes are observed in plasma levels, secretion patterns, and clearance of various hormones in obese individuals *(Álvarez-Castro 2011)*. Alongside a chance association, a direct relationship between "Thyroid and Obesity" has been hypothesized *(Biondi 2010, Marzullo 2010, Rotondi 2011, Laurberg 2012, Pearce 2012, Duntas 2013).*

Thyroid hormone is indeed an important determinant of energy expenditure and contributes to appetite regulation. On the other hand, secretory products from the adipose tissue act on the central nervous system (CNS) to inform on the quantity of energy stores, and this may have an impact on the activity of the hypothalamus–pituitary–thyroid axis *(Biondi 2010, Rotondi 2011, Duntas 2013)*.

Moreover, research has revealed the existence of a positive correlation between adiposity parameters and serum thyrotropin (TSH) and triiodothyronine (T3) in euthyroid individuals, suggesting a possible influence of obesity on the functioning of the hypothalamus-pituitary-thyroid axis and the activity of deiodinases *(Bétry 2015, Kitahara 2012, Lambrinoudaki 2015, Muscogiuri 2013, Ren 2014)*.

Many attempts have been made to treat obese euthyroid subjects with thyroid hormones and/or their analogs in the effort to stimulate energy expenditure, especially during regimens of dietary restriction *(Biondi 2010)*. The matter is a complex one, and it is

further amplified by the fact that obese patients may display alterations in their thyroid function tests, thus raising the question of whether a specific substitution treatment is advisable.

Changes in thyroid function, even within the normal range, may contribute to the worsening of metabolic complications and the development of diseases in the thyroid gland. In this context, it is worth mentioning that high levels of TSH and T3 have been positively associated with components of metabolic syndrome, and specifically higher serum TSH levels have been linked to increased risk of thyroid cancer *(Marcello 2014, Oh 2013, Roef 2014, Taylor 2013, Wang 2016)*.

This section aims to explore the crosstalk between obesity and thyroid function, in particular:

- the association between thyroid function and metabolic phenotype in euthyroid obese individuals
- the effects of early weight loss on the relationship between thyroid function and resting energy expenditure in subjects with severe obesity
- the role of substitution with L-T4 therapy on body composition, REE and glucose-lipid metabolism hypothyroid in severly obese patients

The impact of the metabolic phenotype on thyroid function in obesity

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Abstract

Background. Obesity is known to promote mild hyperthyrotropinaemia by unknown metabolic mechanisms. This investigation aimed to explore the association between thyroid function and metabolic phenotype in euthyroid obese individuals. Retrospective, cross-sectional study. Tertiary care center.

Methods. 952 euthyroid obese individuals referred to our Institution for obesity. Serum levels of TSH, FT4, glucose, insulin and HbA1c levels, lipid profile, liver function and proinflammatory indices were measured. Resting energy expenditure was assessed by indirect calorimetry and body composition by bioimpedance analysis.

Results. On admission, 306 patients had previously diagnosed diabetes mellitus on treatment with metformin, while 113 patients were diagnosed with incident diabetes mellitus. Serum TSH levels were similar between metformin-treated diabetic subjects and metformin-untreated subjects, while FT4 was slightly but significantly higher in the former. Analysis stratified by TSH categories found no effect of metformin-treated diabetes mellitus on TSH levels. Interestingly, obese patients with incident diabetes showed lower TSH levels than normoglycaemic ones. In correlation studies on the whole dataset, an association related TSH to BMI and total cholesterol levels, which was lost upon adjustment for individual confounders. FT4 levels were found to be inversely related to BMI, insulin resistance and triglycerides, while being directly associated with HDL-cholesterol levels. These correlations remained unaltered after controlling for individual confounders. In multivariate linear regression analysis, TSH was associated with FT4, total cholesterol and BMI values. Significant predictors of FT4 were constituted by previously diagnosed diabetes mellitus, BMI, TSH and age.

Conclusions. In euthyroid obese subjects, FT4 seems more closely related than TSH levels to parameters of cardio-metabolic risk. TSH levels did not differ between metformin-treated and untreated subjects, while they were lower in patients with incident diabetes mellitus compared to normoglycaemic ones.

Background

Thyroid disorders impact body weight in multiple ways, and hypothyroidism is traditionally claimed as a contributor of weight accrual via altered metabolic efficiency, water retention, decreased lipolysis *(Santini 2014)*. Nonetheless, case-control studies and epidemiological surveys revealed that TSH levels tend to follow body weight accrual as well as development of obesity and insulin resistance, independent of hypothyroidism *(Knudsen 2005, Michalaki 2006, De Pergola 2007, Rotondi 2009, Marzullo 2010, Souza De Moura 2011, Kitahara 2012, Taylor 2013)*. The causal mechanism underlying this link remains unidentified, yet a compensatory response operated by the hypotalamo-pituitary unit via leptin and directed to counteract weight accrual, is very likely involved *(Santini 2014, Reinehr 2010)*.

Obesity, type 2 diabetes mellitus (T2DM) and thyroid diseases are the most frequent endocrine disorders and often coexist in the same individual *(Duntas 2011)*. Following an original case study *(Vigersky 2006)*, cross-sectional and prospective studies have shown that the antidiabetic drug metformin can decrease TSH levels in patients with subclinical and/or overt hypothyroidism, while other antidiabetic agents do not yield such effect *(Isidro 2007, Cappeli 2009, Rotondi 2011, Fournier 2014, Diez 2013)*. In subsequent studies, it has been clarified that metformin can alter TSH levels also in euthyroid subjects if TSH levels lay in the mid-high normal range (i.e., >2.5–3.0 mIU/L) *(Cappelli 2012, Karimifar 2014, Santos-Palacios 2013)*.

Considering the metabolic derangement commonly associated with obesity as well as the frequent use of metformin in overweight patients owing to its insulin-sensitizing effect and potential reduction of body weight *(Levri 2005)*, we aimed at assessing the impact of the metabolic phenotype, metformin-treated diabetes mellitus, and incident diabetes mellitus on thyroid function in euthyroid obese patients, and thus further investigating the potential thyroidal determinants of cardio-metabolic risk in this setting.

Methods

This study enrolled 952 obese patients [M/F = 410/542, median age 58 (IQR 52–66) years, median BMI 45 (IQR 41.4–49.4) kg/m^2] referred to our institution for work-up and rehabilitation of obesity and its comorbidities. Subjects included in the current study were participants of the TONDO study (T2DM of new diagnosis in obesity), an ongoing study initiated in 2012 and designed to investigate the relationship between glucose abnormalities and indices of organ damage in subjects with uncomplicated and complicated obesity *(Bellan 2014)*, and were included according to inclusion criteria and availability of complete thyroid function assessment. Written consent was obtained from all patient, after full explanation of the purpose and nature of the study. The investigation was approved by the local ethical committee, functioning according to the 3rd edition of the Guidelines on the Practice of Ethical Committees in Medical Research. The current cohort included: (1) 646 euthyroid obese subjects [group OB-Eu; males/females = 283/363, median age 55 (IQR 48–65) years, BMI 43.7 (IQR 40–48.9) kg/m^2], 113 of whom were diagnosed on admission with T2DM based on fasting glycaemia and/or HbA1c levels; (2) 306 euthyroid obese subjects with T2DM on treatment with metformin for at least 6 months [group OB-EuM; males/females, 127/179; median age 58 (IQR 50–65) years; BMI 45.6 (IQR 41.4–49.6) kg/m^2; median diabetes duration 8.1 years (IQR 6.9–9.4)]. Exclusion criteria were age below 18 or above 90 years; previous or current use of medications potentially interfering with thyroid function (such as amiodarone, steroids or lithium carbonate therapy); previous or current treatment with levo-thyroxine; known autoimmune diseases (including thyroid) and/or hypothyroidism; type 1 diabetes mellitus; pregnancy; liver or kidney disease. In case of TSH value >4.5 mIU/L, a condition not mandatorily indicative of thyroid dysfunction in obesity, patients underwent a preliminary thyroid screening inclusive of re-analysis of TSH and measurement of fT3, fT4 and anti-thyroid antibody titer, to exclude true thyroid hypofunction. Screening test included glucose and insulin levels, lipid profile, indices of liver function and proinflammatory state, in fasting conditions. Patients were not prescribed diet therapy, dietary supplements, or antiobesity compounds for at least 3 months prior to entering the study.

Body measurements

All subjects underwent body measurements wearing light underwear, in fasting conditions after voiding. Weight and height were measured to the nearest 0.1 kg and 0.1 cm, respectively. BMI was expressed as body mass (kg)/height $(m)^2$. Obesity was defined for any BMI over 30 kg/m^2 *(WHO 2000)*. Waist circumference was measured midway between the lowest rib and the top of the iliac crest after gentle expiration; hip was measured as the greatest circumference around the nates. Anthropometric data were expressed as the mean of two measurements.

Fat mass and free fat mass, expressed as percentage of total body mass, were assessed by bio-impedance analysis (BIA, 101/S Akern; Florence, Italy) the morning after overnight fasting and after voiding. The two vector components of impedance (i.e. resistance and reactance) were obtained by single measurements; before each testing session, the external calibration of the instrument was checked with a calibration circuit of known impedance value. The mean coefficient of variation was 1% for within-day and 3% for weekly intraindividual measurements in the steady-state condition in either site and 2% for interoperator variability.

Resting energy expenditure (REE) was expressed in kilocalories per 24 h and determined in a thermoregulated room (22–24°C) by computed open-circuit indirect calorimetry, measuring resting oxygen uptake and resting carbon dioxide production by a ventilated canopy (Sensormedics, Milan, Italy) at 1-min intervals for 30 min and expressed as 24 h value. Predicted REE was calculated by the Harris–Benedict formula and allowed to test for metabolic efficiency.

Laboratory tests

Insulin resistance was calculated by the homeostatic model of insulin resistance (HOMA-IR) index: insulin (mIU/L) × [glucose (mmol/L)/22.5]. A HOMA-IR value greater than 2.0 was considered indicative of insulin resistance, as obtained in a sample of the Italian population *(Bonora 2000)*. The homeostatic model of β cell function (HOMA-B) was used to describe the functionality of pancreatic beta cells and calculated using the following formula: 20 [insulin (mIU/L)/glucose (mmol/L) − 3.5]. HbA1c levels were determined in

290 patients of the metformin-untreated group and all metformin-treated patients. ADA recommendations for 2012 *(ADA 2012)* were used for the definition of glucose metabolism and T2DM based on fasting plasma glucose (FPG) and glycated hemoglobin (HbA1c), as follows: normal FPG if <5.6 mmol/L; impaired FPG (IFG) if FPG was 5.5–6.9 mmol/L; T2DM if FPG was ≥7.0 mmol/L on 2 days apart. HbA1c values of 5.7 and 6.5% were considered as the threshold of normal glucose metabolism and T2DM, respectively. Undiluted serum samples were assayed for fT4 and TSH using an automated chemiluminescence assay system (Immulite 2000; DPC, Los Angeles, CA). The principle of the method is a two-site, solid-phase chemiluminescent immunometric assay or competitive immunoassay. Normal values for TSH are 0.4–4.5 mIU/L, and for fT4 102.9-244.5 nmol/L. Insulin levels were measured by immulite. Glucose, total cholesterol, high-density (HDL) and low-density lipoprotein (LDL) cholesterol, and triglycerides were measured by enzymatic methods (Roche Diagnostics, Mannheim, Germany). Fibrinogen levels were determined with the Clauss methodology by Hemosil assay (IL Coagulation System, Instrumentation Laboratory, Bedford, MA). Ultrasensitive C-reactive protein (CRP) was measured by CRP (latex) HS Roche kit, having sensitivity of 0.03 nmol/L, intraassay and interassay CVs of 2.51–5.35 and 4.25–5.79%, respectively, as reported by the manufacturer. Alkaline phosphatase, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were assayed according to the International Federation of Clinical Chemistry (IFCC), without pyridoxal-5'-phosphate, using the Cobas Integra 800 (Roche Diagnostics). Gamma-glutamyltranspeptidase (GGT) was measured by enzymatic colorimetric test using Roche/Hitachi 904/911/912/917/modular (Roche Diagnostics).

Statistics

Statistical analysis was performed using SPSS version 18 (Somers, NY, USA) on log transformed data to correct for the non-Gaussian distribution obtained by the Shapiro–Wilk test. Values are expressed as medians and interquartile ranges. Mann–Whitney test was used for comparison between subgroups. Spearman's correlation analysis and the Chi square were used to identify significant associations between variables of interest. The role of non-collinear variables on TSH and fT4 levels was tested by stepwise multiple regression analysis using as independent variables age, gender, BMI, use of metformin,

total cholesterol and fT4 or TSH, depending on whether TSH or fT4 levels were analyzed, respectively. HOMA-IR and waist circumference were employed as additional covariates in this model after exclusion of potential collinear variables. Logistic regression analysis to test the effect of metformin-treated diabetes mellitus and other variables of interest on the levels of thyroid parameters. Statistical significance was set at 5%.

Results

A summary of anthropometric and biochemical data are reported in Table 1 (pag. 206).
BMI values were comprised between 30–73.6 kg/m^2, and were >40 kg/m^2 in 78.8% (750 patients), >35–39.9 kg/m^2 in 17.8% (172 patients), and >30–34.9 kg/m^2 in 3.1% of cases (30 patients). The metformin-treated diabetic subgroup exhibited greater BMI, waist circumference and fat mass than their counterpart. Both subpopulations were, however, severely insulin resistant and HOMA-IR exceed the normal threshold of 2 (WHO 2000) in 81.6% of metformin-untreated and 87% of metformin-treated diabetic patients. Ninety-four metformin-treated diabetic subjects were on statins at the time of the study, which likely explained the differences in total and LDL-cholesterol noted between groups.
Analysis of thyroid function showed a mild increase in TSH levels in a subset of 30 patients (5.6%). None of these harbored any increase in anti-thyroid antibody titer or abnormal free thyroid hormone levels. Table 1 (pag. 206) illustrates similar TSH levels between metformin-treated diabetic patients and metformin-untreated subjects. Based on the TSH reducing effects previously found to be elicited by metformin, our attention was mainly focused on TSH values in metformin users and non-users, yet data dichotomization by median TSH levels o 1.72 mIU/L showed a comparable distribution of metformin users between top and bottom TSH bearers (24.3 and 23.2%, respectively), with results being equivalent if TSH was stratified by quartiles (Figure 1, pag. 209) or logistic regression analysis (Table 2, pag. 207).
Oppositely, analysis of fT4 levels showed significantly higher fT4 levels in metformin-treated diabetic subjects compared to metformin-untreated ones. Interestingly, when a subset of 113 metformin-untreated obese patients with incident diabetes mellitus was considered separately, these patients exhibited lower TSH [1.50 (IQR 1.12–2.01) vs. 1.80 (IQR 1.24–2.51) mIU/L; p=0.02] and greater abdominal obesity [waist, 132 (IQR 121–141)

vs. 114.7 (IQR 100.4–132.5) cm; p<0.001] than the normoglycaemic counterpart, devoid of differences in fT4 levels [11.1 (IQR 10.2–12.4) vs. 11.3 (IQR 10.2–12.6) pg/mL] or BMI values [44.5 (IQR 40.3–49.3) vs. 43.5 (IQR 39.5–48.2) kg/m^2]. Gender stratification showed no differences in TSH and fT4 levels (data not shown).

Bivariate regression analyses on the whole dataset depicted a significant negative correlation between TSH and fT4 levels (rho=−0.091, p=0.005), with each being weakly correlated in opposite direction with BMI (TSH: rho=0.078, p=0.02; fT4: rho=−0.074, p=0.02) and waist circumference (TSH: rho=0.063, p=0.06; fT4: rho=−0.066, p=0.04). The relationship between TSH and fT4 persisted after controlling for age, BMI, gender, HOMA-IR and the use of metformin (r=0.088, p=0.008). No correlation was observed between TSH and age. In sub-group analysis, patients aged >70 year (103 cases, 10.8%) showed comparable TSH levels with the remainders (1.68 mUI/L [IQR 1.25–2.46] vs 1.72 mUI/mL, [IQR 1.21–2.47]); the possibility that this represented a 'survivor' sample of patients was hinted by the finding of a slightly healthier metabolic profile when compared to the younger counterpart, i.e. lower levels of total and LDL cholesterol, triglycerides, and HbA1c (data not shown). When thyroid function was plotted against serum parameters of cardiovascular risk in the population as a whole, TSH was only associated with total cholesterol (rho=0.080, p=0.01). Of note, fT4 levels were inversely related to triglycerides (rho=−0.091, p=0.005), insulin (rho=−0.079, p=0.02) and HOMA-IR (rho=−0.083, p=0.01), and directly to HDL-cholesterol (rho=0.106, p=0.001). In testing the relationship between thyroid function and cardio-metabolic variables after controlling for potential confounders (i.e. age, gender, BMI, incident diabetes mellitus, use of metformin, use of statins, smoking), only fT4 remained correlated with age, BMI and HDL-cholesterol, while the correlation with triglycerides approached statistical significance (Table 3, pag. 208). No relationship was seen between thyroid function and C-reactive protein, fibrinogen and liver function tests, but with alkaline phosphatase (TSH: rho=−0.098, p=0.002; fT4: rho=0.070, p=0.03). Likewise, no association related thyroid function to REE and metabolic efficiency, as well as BIA-derived parameters of body composition (data not shown).

In multivariate linear regression analysis, TSH was best predicted by fT4 (standardized β=−0.079, p=0.015), total cholesterol (standardized β=0.074, p=0.02) and BMI (standardized β=0.065, p=0.04). Replacement of BMI with waist circumference did not

reach statistical significance. When FT4 levels were tested as the dependent variable, they were predicted by TSH (standardized β=−0.84, p=0.01), metformin-treated diabetes mellitus (standardized β=0.087, p=0.008), BMI (standardized β=−0.83, p=0.012) and age (standardized β=−0.80, p=0.015). When waist circumference replaced BMI, it also entered the regression equation (standardized β=−0.077, p=0.021). HOMA-IR did not enter the regression equation both when TSH and fT4 levels were tested.

Discussion

Obesity is conventionally regarded as the result of unbalanced calorie intake and impaired energy expenditure acting on a predisposed genetic setting. A common explanatory model encompasses the lipostatic regulation system, in which energy stores generate signals that are compared with targets encoded in the brain, and differences between these drive our food intake levels, activity patterns, and resting and active metabolisms *(Speakman 2004)*. Although obesity predisposes to hyperthyrotropinaemia and reductions in fT4 levels *(Santini 2014, Knudsen 2005)*, TSH levels can still provide a peripheral index of thyroid activity also in the obese state *(Tagliaferri 2001)*. So far, analyses on the metabolic correlates of thyroid function in obesity have provided little evidence, or were conducted in small cohorts so as to draw definitive conclusions *(Santini 2014, Knudsen 2005)*. In this regard, a potential confounder is constituted by the antidiabetic agent metformin, based on its ability to reduce TSH levels in subjects with hypothyroidism and in those with euthyroidism harboring normal-high TSH levels *(Duntas 2011, Vigersky 2006, Isidro 2007, Cappelli 2009, Rotondi 2011, Cappelli 2012, Karimifar 2014, Santos-Palacios 2013)*. Metformin acts as insulin-sensitizer in multiple ways, such as by decreasing hepatic glucose production through inhibition of the mitochondrial respiratory-chain complex 1, by activating the cellular metabolic sensor AMPK, by increasing glucagon-like peptide 1 (GLP-1) levels, as well as by inducing islet incretin receptor gene expression *(Zhou 2001, Gunton 2003, Maida 2011, Lim 2010)*. The exact mechanisms linking metformin effects to variations in pituitary TSH secretion are currently unknown, but possibly involve inhibition of hypothalamic AMPK, counteraction of central T3 effects, dopaminergic effects and/or inhibition of pituitary TSH secretion *(Duntas 2011, Lopez 2010, Labuzek 2010)*. In the current study, first of all we documented

comparable TSH levels between obese patients treated and not treated with metformin. This result was confirmed by multiple statistical approaches. Oppositely, a slight yet significant increase in fT4 levels was seen in metformin-treated subjects compared with their untreated counterpart. This latter finding contrasts with the observation that metformin leaves unaltered free thyroid hormone levels *(Lupoli 2014)*, and possibly reflects an effect played by obesity on thyroid hormone metabolism either mediated by type 2 deiodinase activity, TH binding to TBG, or thyroxine degradation. However, we do not exclude that such finding simply results from metabolic differences existing between subgroups, since metformin-treated subjects were slightly older and mildly more insulin resistant, showed a more central distribution of body fat and higher BMI compared to the metformin-untreated counterpart. Along this line of reasoning, the positive correlation between fat mass and fT4 found by others *(Roef 2012)* supports the hypothesis that abdominal adiposity may increase free thyroid hormone. In addition, diabetes mellitus can increase per se fT3 inactivation to rT3 and decrease T4 conversion to T3 due to lower type 2 deiodinase activity *(Moura Neto 2013)*, while metabolic syndrome can lead to higher fT4 levels in obese subjects *(Tarcin 2012)*. Our obese patients with incident diabetes mellitus were found to harbor lower TSH compared with their metformin-untreated counterpart. In this subgroup, individual analysis found no potential case of subclinical dysfunction and no significant metabolic impact was documented in correlation analyses likely due to the sample dimension and the overall high rate of severe obesity in our study (nearly 80%). Further investigations in larger datasets are warranted to verify the clinical significance of this observation.

Together, our results pinpoint potentially different metabolic roles for TSH and fT4 in the obese setting. In general, TSH was associated with increasing BMI and total but not HDL cholesterol levels. These associations disappeared after controlling for individuals' confounders. Oppositely, fT4 levels showed an inverse relation with BMI, insulin, HOMA-IR, triglycerides, in conjunction with a direct association with HDL-cholesterol. fT4 associations with the metabolic phenotype remained significant after adjustment for potential confounders. This leads us to speculate that fT4 reflects better than TSH the cardio-metabolic risk of obesity: according to this view, TSH levels mirror the obese phenotype, while fT4 levels act as a proxy of the metabolic status related to the obese phenotype. Previous studies in morbidly obese patients found an association of increasing

TSH, and less significantly decreasing FT4, with insulin and insulin resistance *(Michalaki 2006, Iacobellis 2005, Ambrosi 2010)*, yet none was controlled for individual confounders. On the other hand, population studies related low fT4 more tightly to cardio-metabolic indices (BMI, insulin resistance, lipid profile and intima media thickness) than high TSH levels *(Roos 2007, Dullaart 2007, Kim 2009, Mehran 2014)*. In a Korean study on 6241 non diabetic euthyroid subjects, those with the lowest fT4 quartile had twice the risk for insulin resistance as compared to those in the highest quartile after adjustment for age, sex, metabolic, and life style factors *(Shin 2014)*. Nevertheless, the NHANES 2007–2008 analysis on 3114 euthyroid healthy men and women found that only TSH levels, and to a lesser degree fT3, were correlated with BMI and waist circumference, while fT4 levels were not *(Kitahara 2012)*. Likewise, the Asklepios Study on 2315 healthy euthyroid middle-aged men and women found that a higher fT3–fT4 ratio (index of conversion from fT4 to fT3) was predictive of an unfavorable metabolic profile *(Roef 2014)*. Differences in prevalence and degree of obesity, fat partition, genetic background, and insulin sensitivity may contribute to explain the observed discrepancies.

Conclusions

In conclusion, fT4 levels appeared to be related to the overall metabolic phenotype of obese patients, while the metabolic impact of TSH was less prominent. Incident diabetes seems associated with a reduction in TSH results, yet further studies in larger samples of euthyroid obese patients are needed to detail the individual clustering of TSH and fT4 levels with metabolic features in this setting.

Tables

Table 1. Summary of anthropometric and biochemical data obtained in the whole study population, in the euthyroid metformin-treated obese diabetic subpopulation (OB-EuM) and the euthyroid obese metformin-untreated subpopulation (Ob-Eu).

Variables	Whole population (n = 952)	OB-EuM (n = 306)	Ob-Eu (n = 646)	p
Males/females	410/542	127/179	283/363	0.5
Age (years)	56 (49–65)	58 (50–65)	55 (48–65)	0.07
BMI (kg/m^2)	44.4 (40.5–48.9)	45.6 (41.4–49.6)	43.7 (40.0–48.7)	0.003
Weight (kg)	117.8 (102.8–133.5)	118.3 (106.0–134.0)	117.3 (101.6–133.1)	0.09
Height (cm)	161.8 (155–171)	161 (155–170)	162 (155–171)	0.3
Waist (cm)	129 (119–138)	131 (122–140)	127 (116–137)	<0.001
Waist-to-hip ratio	0.96 (0.89–1.92)	0.97 (0.91–1.04)	0.94 (0.88–1.02)	<0.001
REE (kcal/day)	1909 (1651–2247)	1963 (1740–2280)	1884 (1598–2233)	0.003
Fat mass (%)	46.3 (41.0–51.8)	47.3 (41.9–52.6)	45.7 (40.4–51.3)	0.009
Body water (%)	39.7 (36.2–44.2)	39.5 (36.3–43.9)	39.8 (36.2–44.4)	0.7
TSH (mIU/L)	1.72 (1.21–2.47)	1.72 (1.19–2.45)	1.72 (1.22–2.47)	0.7
fT4 (nmol/L)	146.3 (132.5–162.1)	151.9 (135.1–163.5)	145.4 (131.3–162.2)	<0.001
Insulin (pmol/L)	100.7 (65.9–145.8)	98.6 (62.5–150.0)	102.1 (68.1–143.1)	0.5
Glucose (mmol/L)	5.77 (5.05–7.21)	7.38 (6.05–8.99)	5.38 (5.38–6.22)	<0.001
HbA1c (%)	6.6 (6.0–7.9)	7.5 (6.5–8.7)	6.1 (5.7–6.7)	<0.001
HOMA-IR	4.0 (2.5–6.1)	4.7 (2.9–7.2)	3.7 (2.3–5.6)	<0.001
HOMA-B	125.2 (72.1–214.1)	76.3 (42.6–133.1)	152.0 (97.1–248.3)	<0.001
Cholesterol (mmol/L)	5.10 (4.38–5.80)	4.79 (4.01–5.44)	5.26 (4.66–5.98)	<0.001
LDL (mmol/L)	3.12 (2.49–3.76)	2.75 (2.16–3.39)	3.30 (2.70–3.93)	<0.001
HDL (mmol/L)	1.09 (0.91–1.35)	1.04 (0.85–1.27)	1.11 (0.93–1.37)	<0.001
Triglycerides (mmol/L)	1.59 (1.23–2.18)	1.73 (1.31–2.37)	1.55 (1.20–2.09)	<0.001
AST (µkat/L)	0.35 (0.28–0.48)	0.35 (0.28–0.50)	0.35 (0.30–0.48)	0.8
ALT (µkat/L)	0.45 (0.32–0.70)	0.43 (0.32–0.70)	0.45 (0.32–0.70)	0.8
γGT (µkat/L)	0.48 (0.32–0.75)	0.50 (0.33–0.87)	0.47 (0.30–0.72)	0.07
ALP (µkat/L)	1.64 (1.14–3.02)	1.25 (1.04–1.65)	2.27 (1.25–3.35)	<0.001
CRP (nmol/L)	5.71 (2.86–10.48)	5.71 (2.86–11.43)	5.71 (3.81–10.48)	0.5
Fibrinogen (µmol/L)	11.64 (10.17–13.38)	11.44 (9.91–13.17)	11.76 (10.29–13.52)	0.04

HbA1c levels in Ob-Eu refer to measurement in a subgroup of 290 patients. Data are expressed as medians (with interquartile range in parentheses). Comparison between populations was performed by Mann–Whitney test on log-transformed data and χ2 test

BMI body mass index; REE resting energy expenditure; HOMA-IR homeostatic model of insulin resistance; HOMA-B homeostatic model of β cell function; LDL low density lipoprotein; HDL high density lipoprotein; AST aspartate aminotransferase; ALT alanine aminotransferase; γGT gamma glutamiltransferase; ALP alkaline phosphatase; CRP C-reactive protein

Table 2. Logistic regression analysis on values of TSH and BMI dichotomized by median values (0 = bottom values; 1 = top values) in the population as a whole.

Variables	**TSH**	**BMI**
Age	0.98 (0.96–0.99)	0.99 (0.98–1.01)
Female gender	1.08 (0.59–1.73)	*5.67 (3.61–8.90)*
TSH	–	1.05 (0.89–1.24)
BMI	0.97 (0.94–1.01)	–
REE	1.00 (0.99–1.00)	1.003 (1.002–1.003)
HOMA-IR	1.01 (0.99–1.02)	0.99 (0.98–1.01)
Use of metformin	1.09 (0.78–1.52)	1.29 (0.90–1.85)

Odds ratio and 95 % confidence intervals are displayed. BMI body mass index; REE resting energy expenditure; HOMA-IR homeostatic model of insulin resistance. Use of metformin was categorized as 0 = no use of metformin, 1 = use of metformin. Significant values are in italics.

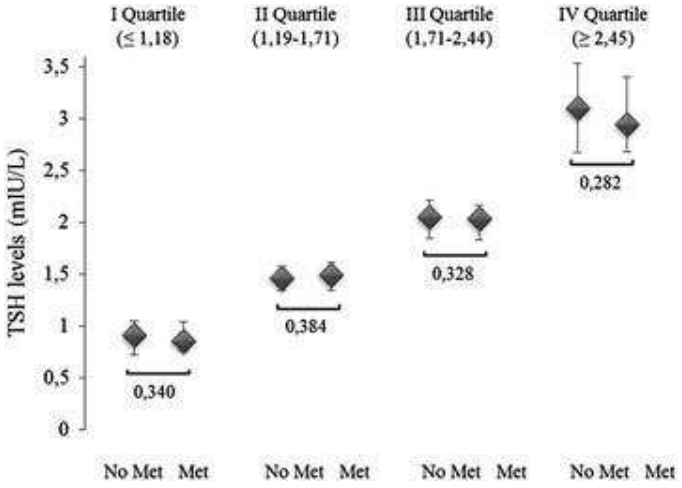
Table 3. Correlation analysis between thyroid function and phenotypic variables of interest in the obese group as a whole, after controlling for age, gender, BMI and use of metformina.

Variables	**TSH**		**fT4**	
	r	**p**	**r**	**p**
Age	−0.05	*0.1*	**−0.08**	***0.01***
BMI	0.05	*0.09*	**−0.09**	***0.006***
Waist	0.04	*0.3*	0.02	*0.5*
Percent fat mass	**0.09**	***0.02***	0.07	*0.06*
Cholesterol	0.506	*0.09*	−0.02	*0.5*
HDL	0.03	*0.3*	**0.13**	***0.001***
LDL	0.04	*0.3*	−0.05	*0.1*
Triglycerides	0.05	*0.2*	−0.07	*0.06*
Insulin	0.01	*0.7*	−0.03	*0.3*
HOMA-IR	0.01	*0.8*	−0.04	*0.2*
HOMA-B	0.02	*0.5*	0.01	*0.7*
AST	0.03	*0.4*	−0.07	*0.06*
ALT	−0.003	*0.9*	−0.003	*0.9*
γGT	0.004	*0.2*	−0.03	*0.4*
Alkaline phosphatase	**−0.13**	***0.001***	**0.09**	***0.02***

Italic values indicate p-values
Underlined values indicate significance
Italic underlined values indicate statistically significant p-values
Age and BMI were omitted as covariates when measured as independent variables

Figures

Figure 1. Comparison of TSH quartiles between obese euthyroid obese patients with diabetes and treated with metformin (Met) and those without diabetes and not treated with metformin (No Met). Medians (diamonds) and interquartile ranges (lines) are displayed.



Early weight loss reactivates the relationship between thyroid hormones and resting energy expenditure in severe obesity

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Abstract

Purpose. The effects of early weight loss on the relationship between thyroid function and resting energy expenditure (REE) in subjects with severe obesity is unknown.

Methods. To explore the early effects of weight loss on this association, 100 severely obese euthyroid inpatients underwent a 4-wk multidisciplinary moderately hypocaloric dieting program inclusive of measurements of thyroidal parameters, REE and body composition.

Results. No association related thyroid function to REE at baseline. After 4-wk dieting, weight loss was modest but significant (-5.5%, $p<0.0001$ vs. baseline) and paralleled by reductions in REE (-4.1%, $p<0.001$), fat-free mass (-3.7%, $p<0.001$) and fat mass (-1.6%, $p<0.05$). Early thyroidal changes after weight loss were marginal but significant for TSH (-6.3%), FT3 (-3.3%) and FT4 levels (+3.9%, $p<0.001$ for all). After weight loss, REE resulted associated with FT3 ($r=0.24$, $p<0.05$) and FT4 ($r=0.42$, $p<0.001$) via interactions with fat-free mass ($F=13.4$ and 9.03, respectively; $p<0.001$ for both). In multivariate analysis, no thyroidal variable entered the regression equation for REE response to weight loss.

Conclusions. Early weight loss reactivates the relationship between thyroid and energy balance. This prompt, and likely non-adaptive, response may physiologically act to regulate long-term management of severe obesity depending on starting body composition.

Background

Physiological and environmental factors are known to influence the normal function of the hypothalamo-pituitary thyroid (HPT) axis, including iodine intake *(Rasmussen 2002)*, aging *(Surk 2007)*, reproductive stages *(Negro 2014)*, ethnic and geographical determinants *(Spencer 2007)*, several medications *(Aoki 2007)* and smoking *(Makepeace 2008)*. Increasing attention has been dedicated to the ability of obesity to promoting a shifting of serum TSH concentrations toward normal-high or slightly supra-normal levels, while influencing subtle increments in T3 levels and reductions in FT4 levels *(Biondi 2010)*. The mechanisms linking adipose tissue expansion to a change thyroidal parameters likely originate from a compensatory response operated by the hypotalamo pituitary unit to counteract adiposity via leptin-mediated effects *(Santini 2014)*. In addition to assessing the coexistence of thyroid disorders, analysis of thyroid function in the severely obese setting can be useful to rule out the possible impairment of resting energy expenditure (REE) as a potential contributor of obesity *(Tagliaferri 2001)*. This inference is based on the evidence that regulating thermogenesis is one of the major tasks of THs in adult humans, which involves direct and indirect effects on cellular processes controlling triiodothyronine-responsive energetic processes *(Kim 2008)*. Opposed to the classical view that most effects of THs on energy homeostasis are peripheral, current perspectives indicate that THs can centrally affect energy metabolism by interacting with global energy sensors *(López 2013)*. Upon body weight loss, it has long been acknowledged that REE decreases as a consequence of a reduction of fat free mass (FFM) *(Ravussin 1985)*. Likewise, weight loss blunts serum TSH concentrations, 24-hr TSH fluctuations and the TSH response to thyrotropin releasing hormone (TRH), as well it promotes a decrease in FT3 concentrations and the increment of FT4 levels *(Santini 2014)*. The functional combination of these changes is meant to be primarily adaptive, and is partly leptin-mediated *(Rosenbaum 2002)*. Age, clinical setting, type and duration of intervention, and individual confounders can influence the adaptive modifications of the relationship between the HPT axis and energy homeostasis during weight loss *(Froidevaux 1993, Reinehr 2002, Sari 2003, Kok 2005, Agnihothri 2014)*.

Because non-adaptive effects of caloric restriction on such relationship have been scarcely characterized, the current study was undertaken to explore the potentially early

mechanisms relating changes in thyroid function to REE in function of short-term weight loss by using an inpatient approach. To this aim, a 4-week timeframe was used for the current investigation, based on the demonstration that 6-8 weeks are required to reset the HPT axis *(Fish 1987, Garber 2012)*. We thus aimed to profile early thyroidal and REE modifications in euthyroid individuals with severe obesity, with the intention of shedding new light on non-adaptive associations between weight loss, thyroid function and energy homeostasis, and possibly to inform on long-term obesity management decisions.

Methods

The study population was constituted by 100 euthyroid obese patients (BMI >30 kg/m^2), classified as severely obese (52 females/48 males; mean age, 40.4±12.7 yr; mean BMI, 45.1±4.8 kg/m^2, BMI range, 40-61 kg/m^2), who were recruited after signing an informed consent upon admission to our Institution for diagnostic workup and rehabilitation for severe obesity. Following baseline assessment, all participants underwent a 4-week inpatient study consisting of multidisciplinary weight loss program entailing the following: a) personalized diet, daily monitored by a dietician, formulated according to the Italian recommended daily allowances (Italian Society for Human Nutrition), entailing an energy intake corresponding to the 75% of the measured REE; b) aerobic physical activity program, including two 30 min sessions/day of cycle erogometer pedaling, treadmill walking and stationery rowing, carried out for 5 days/week. The intensity of exercise was set at an average heart rate between 60% and 80% of the individual's maximum heart rate; c) psychological and nutritional counselling. To reduce potential for selection bias, an effort was made to maintain equal distributions in gender, individualized dietary intake, energy expenditure and counseling. Each part of the study was conducted under skilled medical surveillance and nursing. To reduce differences in performance, medical and nursing staff in charge were unaware of patients participation to the study, and the ancillary treatment and diagnostic investigations received by the study group paralleled that of regular inpatients. The study was approved by the Ethic Committee of the Istituto Auxologico Italiano. Study procedures were performed in accordance with the Helsinki Declaration of 1975 as revised in 1983. To reduce detection bias, none of the study participants suffered from thyroid disease or had been previously treated with

medications potentially interfering with thyroid function. Exclusion criteria included endocrine obesity, autoimmune or chronic inflammatory disorders, type 1 diabetes mellitus (T1DM) and T2DM, chronic obstructive pulmonary disease, history of neoplasms or degenerative diseases, previous chronic steroid treatment, kidney or cardiac disorders, liver disease. No patient was undergoing pharmacological therapies at the time of the study, and body weight had been stable for at least three months prior to study entry. Baseline testing was performed at 08.00 h in fasting conditions and after voiding, 3 d after admission while patients were fed a balanced diet (30% lipids, 50% carbohydrates, and 20% proteins). Neither supplements nor anti-obesity therapies were used during the study period.

Anthropometry, energy expenditure and thyroid ultrasonography

The study protocol included metabolic profiling and thyroid ultrasonography (US) at study entry, while hormonal assessment as well as anthropometry data, body composition and energy expenditure were determined at baseline and at the end of the study. For each patient, three main trajectories of body weight increase were included: child-hood onset obesity, adult-onset obesity and weight oscillations.

All subjects underwent body measurements wearing light underwear, in fasting conditions after voiding. Weight and height were measured to the nearest 0.1 kg and 0.1 cm, respectively. BMI was expressed as body mass (kilograms)/height (meters)^2. Obesity was defined for BMI over 30 kg/m2. Waist circumference was measured midway between the lowest rib and the top of the iliac crest after gentle expiration; hip was measured as the greatest circumference around the nates. Anthropometric data were expressed as the mean of two measurements.

Respiratory quotient (RQ; VO2/VCO2) and REE (kcal/24 h) were determined in a thermo-regulated room (22–24°C) by computed open-circuit indirect calorimetry, measuring resting oxygen uptake and resting carbon dioxide production by a ventilated canopy (Sensormedics; Milan, Italy) at 1-min intervals for 30 min and expressed as a 24-h value.

Predicted REE (pREE; kcal/24 h) was calculated by the Harris-Benedict formula and was employed to calculate the REE/pREE ratio as a proxy of metabolic efficiency, set as 100% *(Marzullo 2004)*. Fat mass and free fat mass were assessed by bio-impedance analysis

(BIA, 101/S Akern; Florence, Italy) the morning after overnight fasting and after voiding. The two vector components of impedance (i.e. resistance and reactance) were obtained by single measurements; before each testing session, the external calibration of the instrument was checked with a calibration circuit of known impedance value. The mean coefficient of variation was 1% for within-day and 3% for weekly intra-individual measurements in the steady-state condition in either site and 2% for inter-operator variability.

Thyroid ultrasonography (US) was performed by one certified operator (AM) using a real-time US device equipped with a linear transducer operating at 7.5 MHz for morphologic study (MyLab Class C, Esaote Biomedica, Genova, Italy). US pattern of thyroid parenchyma was defined for echogenicity based on comparison with neck muscles on transverse section, after excluding the potentially reflecting echoes from isthmus due to the tracheal cartilage. The volume of the thyroid gland was calculated according to the ellipsoid model [length (cm) × width (cm) × depth (cm) × 0.5], and the size of nodules, if present, was measured. Vascularization of the gland and of the nodules was semiquantitatively assessed, as well as the occurrence of micro-calcifications and the spongiform aspect. Combinations of diagnostic signs for thyroid nodules included size >1 cm, microcalcifications, a taller-than-wide shape, irregular borders, hypoechogenicity, and intranodular vasculature.

Laboratory determinations

Thyroid function was tested by analysis of FT4, FT3, TSH, anti-Tg antibodies (TgAb) and anti-TPO antibodies (TPOAb) levels. Undiluted serum samples were assayed using an automated electrochemiluminescence assay system (Cobas 6000; Roche Diagnostics GmbH, Mannheim, Germany). The principle of the method is a two-site, solid-phase chemiluminescent sandwich immunoassay. Normal values were as follows: FT3, 1.8–4.4 ng/L; FT4, 8.0–19.0 ng/L; TSH, 0.27–4.2 mIU/L; TPOAb, less than 35 µU/L; TgAb, less than 40 µU/L. Baseline evaluation 130 included analysis of the lipid panel, glucose and insulin levels. Glucose homeostasis was based on fasting blood glucose and/or glycated hemoglobin (HBa1c) levels according to ADA guidelines *(Cunningham 1991)*, as well as insulin resistance calculated by the homeostatic model (HOMA-IR) index: insulin (mIU/L) x

[glucose (mmol/liter)/22.5]. Blood glucose, lipids and C reactive protein levels were measured by enzymatic methods; a two-site solid-phase electrochemiluminescent sandwich assay was used for insulin levels (Cobas 6000).

Statistical analysis

Data were tested for normality of distribution by the Kolmogorov-Smirnov test and log-transformed when needed, to correct for skewness. Study outcomes were constituted by the interactions relating percent modifications of body weight and biochemical, anthropometric, and body composition variables measured at the end of the 4-wk study period. Parameters of interest were thus considered as values at study entry, at the study end and as percent delta variations over baseline values (Δ). For comparative analyses, paired T test for intra-individual comparisons and ANOVA between subgroups were used. Correlation between analyzed parameters and their deltas were tested by bivariate regression analyses, partial correlation analysis, and ANCOVA by the general linear model to control for interactions. Females were categorized as 0 and males as 1. Stepwise multivariate regression analysis was used to evaluate the independent association of variations in REE, TSH and THs with metabolic or anthropometric variables. Regression models were built on the results of simple and partial regression analyses, and included age, gender (0=female, 1=male), and baseline and deltas values of FT3, FT4, TSH, REE and BMI. β coefficients, related significance values and adjusted R2 values obtained from the models are reported. $P<0.05$ was considered as statistically significant.

Results

Results obtained at baseline and study end are summarized in Table 1 (pag. 220).

At the study entry, 17 patients showed impaired glucose tolerance and 11 others showed incidental T2DM. Gender-stratified analysis (Table 2, pag. 221) displayed older age, stronger metabolic impairment, and higher alcohol intake in men than women. REE correlated with FFM ($r=0.68$, $p<0.001$), male gender ($r=0.54$, $p<0.0001$), BMI ($r=0.31$, $p<0.01$) and percent FM ($r=-0.25$, $p<0.05$). Baseline TSH levels were above normal in two

patients free of thyroid antibodies or abnormal US pattern. There was at this stage no relationship between thyroidal parameters and REE or its derived measures.

At US, thyroid hypoechogenicity was found in 11% of cases, and thyroid nodules were detected in 60% of cases. One of 8 patients undergoing fine-needle biopsy harbored a medullary thyroid carcinoma. Thyroid nodules were associated with TSH ($r=-0.33$, $p<0.001$) and age ($r=0.31$, $p<0.01$). An association was seen between REE and thyroid volume ($r=0.33$, $p<0.01$), which disappeared after controlling for age, BMI and gender.

At the end of the 4-week weight loss program, significant changes in most anthropometric, hormone and metabolic parameters were observed (Tables 1-2, pag. 220, 221). Particularly, variations of thyroidal parameters (Figure 1, pag. 223) were interindividually skewed but overall significant and each associated with respective values recorded at the study entry (Table 3, pag. 222). Marginal associations were observed between ΔTSH and ΔBMI after adjustment for baseline FFM ($r=0.20$, $p<0.05$), as well as between ΔFT3 and baseline BMI and FFM ($r=0.21$ and $r=0.20$, $p<0.05$ for both), while stronger associations were observed between ΔFT4 and ΔBMI ($r=-0.38$, $p<0.001$), and between ΔFT4 and baseline values of FFM ($r=0.32$, $p<0.001$) and percent FM ($r=-0.22$, $p<0.05$).

At odds with the null association found at study entry, weight loss disclosed a positive correlation between REE and FT4 ($r=0.42$, $p<0.001$) as well as FT3 levels ($r=0.24$, $p<0.05$), via significant interaction with the initial FFM (REE and FT3: $F=13.4$, $p<0.0001$; REE and FT4: $F=9.03$, $p<0.001$). After weight loss, REE was also associated with male gender ($r=0.55$, $p<0.0001$), as well as final values of FFM ($r=0.71$, $p<0.0001$), BMI ($r=0.24$, $p<0.05$) and percent FM ($r=-0.33$, $p<0.01$). ΔREE was only marginally associated with ΔBMI ($r=0.22$, $p<0.05$).

In stepwise multivariate regression analyses, ΔREE was best predicted by its baseline values ($\beta=-0.59$, $p<0.001$) and male gender ($\beta=0.28$, $p<0.05$), with an adjusted R2 of 0.23. Analysis of thyroidal parameters found that ΔTSH was best predicted by its baseline values ($\beta=-0.30$, $p=0.003$) and ΔBMI ($\beta=0.20$, $p=0.03$); ΔFT4 was predicted by its baseline values ($\beta=-0.51$, $p<0.001$), male gender ($\beta=0.36$, $p<0.001$) and ΔBMI ($\beta=-0.17$, $p<0.05$); ΔFT3 was predicted by its baseline values ($\beta=-0.58$, $p<0.001$) and male gender ($\beta=0.33$, $p<0.001$). Adjusted R2 values for the ΔTSH, ΔFT4 and ΔFT3 regression models were 0.10, 0.41 and 0.33, respectively.

Discussion

Growing attention has recently focused on the ability of weight loss to restore thyroidal parameters previously disrupted by significant accumulation of adipose tissue *(Santini 2014)*. It remains unclear if changes in REE, a measure of obligatory thermogenesis that promptly decreases upon dieting, may interact with early thyroidal modifications determined by reductions in bodyweight. In the present study, short-term weight loss was found to modify thyroidal parameters in severe obesity and reactivate a relationship between THs and REE via significant interaction with the fat-free mass.

From the standpoint of energy balance and weight control, these results expand to severe obesity the link between thyroid, fat accumulation and energy homeostasis, and further add early improvements of the association between THs and REE to the extensive list of beneficial effects of weight loss in obesity. It has long been known that obesity can disrupt the HPT axis due to compensatory mechanisms likely directed at increasing energy expenditure, while weight loss promotes reductions of TSH and FT3 levels, and, more debatedly, increases in FT4 concentrations *(Santini 2014)*. In dieting obese women, it was previously found that at least 10% weight loss, obtained in 6 months, was needed to significantly reduce TSH levels *(Sari 2003)*. While our results show that a short-term 5.5% weight loss can reduce TSH as well as FT3 and increase FT4 levels, they especially underscore the favorable role of early weight loss on the relationship between REE and thyroid hormones via significant interaction with fat-free mass. This particular circumstance agrees with the established role of fat-free mass as the single best predictor of REE, as well as inter-individual variability in REE, over a broad range of body weights *(ADA 2016)*, while THs may explain 20-25% of REE variability *(al-Adsani 1997)*. In judging the sensitivity of REE to thyroidal changes herein observed, we are inclined to consider our short-term results as non-adaptive, based on the inference that 6-8 weeks are needed to reset the HPT axis *(Fish 1987, Garber 2012)*. Therefore, we hypothesize that even a short-term and overall modest bodyweight loss may act to reactivate the relationship between THs and REE. Moreover, the stronger relationship between several anthropometric parameters as well as REE with changes in FT4 as compared to FT3 confirms previous observations on the predictive metabolic role of FT4 levels obtained in populations with wide BMI ranges *(Johnstone 2005)* as well as with severe obesity

(Marzullo 2016). Responses of REE to small thyroidal variations have not, to our knowledge, been reported before in severe obesity, which affects a minor proportion of the population but accounts for a disproportionate amount of medical illnesses and health care services.

As small reductions in body weight, i.e. 5%, have the potential to significantly influence health of patients with obesity *(Magkos 2016)*, our findings may help to explain the health-related effects of small reductions in bodyweight.

Although our obese patients were selected as euthyroid, 60% showed incidental thyroid nodules, and individuals with thyroid nodules harbored lower TSH, were generally older and had larger thyroids than patients without. However, the observed prevalence of incidental thyroid nodules overlaps that found in the general population *(Russ 2014)*. Because our report provides no mechanistic information, long-term data are needed to better profile the relationship between thyroid function, energy homeostasis and weight loss maintenance. Similarly, as study participants were selected as euthyroid and severely obese, our findings may not apply to people with normal bodyweight or mild obesity, as well as those with thyroid dysfunctions. Nevertheless, we consider the homogeneous study sample and the controlled weight management schedule as points of strength of this study.

Conclusions

In conclusion, a short-term weight loss seems capable of reactivating the favorable relationship between REE and thyroid hormones in severe obesity, via interactions with fat-free mass. How body composition effectively controls thyroid hormone effects on energy expenditure during long-term calorie restriction warrants further investigation, as it could frustrate weight loss attempts of obese individuals.

Tables

Table 1. Baseline data in the obese population obtained at baseline and at the end of the 4-week study, and expressed as percent variation over baseline values (Δ). Significance between the two time points was obtained by paired T test and is depicted as: a, $p<0.05$; b, $p<0.01$; c, $p<0.001$.

Variables	Baseline (n=100)	After 4 weeks (n=100)	Δ
Age (years)	40.4±12.7	-	-
BMI (kg/m^2)	45.1±4.8^c	42.5±4.4	-5.5±1.8
Waist (cm)	128±12.2^c	122.6±11.6	-4.3±1.2
FPG (mg/dL)	99.2±26.9	-	-
Insulin (mIU/L)	22.2±30.3	-	-
HbA1c (%)	5.9±1.2	-	-
HOMA-IR	5.2±6.9	-	-
CRP (mg/dL)	0.9±0.8	-	-
CHO (mg/dL)	201.7±39.1	-	-
HDL (mg/dL)	44.9±11.8	-	-
LDL (mg/dL)	134.1±35.1	-	-
Triglycerides (mg/dl)	153.8±78.9	-	-
TSH (mIU/L)	2.09±0.92^c	1.87±0.91	-6.3±32.2
Free T3 (ng/L)	3.22±0.36^c	3.10±0.33	-3.3±9.7
Free T4 (ng/L)	11.7±1.59^c	12.0±1.52	3.9±12.7
FT3/FT4 ratio	0.28±0.06^b	0.26±0.03	-5.7±13.4
REE (Kcal/24h)	2038±372^c	1936±322	-4.1±10.8
REE/pREE (%)	94.1±11.0^a	91.8±10.2	-0.12±0.5
RQ (VO2/VCO2)	0.78±0.08	0.80±0.07	3.1±12.6
FM (%)	46.7±6.8^a	45.8±6.9	-1.6±6.7
FFM (kg)	67.2±13^c	64.5±12.5	-3.7±7.7

For abbreviations: BMI, body mass index; FPG, fasting plasma glucose; FI, fasting insulin; HbA1c, glycated haemoglobin; HOMA-IR, homeostatic model of insulin resistance; CRP, C-reactive protein; CHO, cholesterol; HDL, high density lipoprotein; LDL, low-density lipoprotein; REE, resting energy expenditure; pREE, predicted REE; REE/pREE, metabolic efficicncy; RQ, respiratory quotient; FM, fat body mass; FFM, fat-free body mass.

Table 2. Data summary obtained at baseline and at the end of the 4-week study in the obese population subgrouped by gender. Significance for between-gender ANOVA at baseline and at the study-end time-points is depicted as: a, $p<0.05$; b, $p<0.01$; c, $p<0.001$. Significance for within-gender paired T test between baseline and study-end time-points is depicted as: d, $p<0.05$; e, $p<0.01$; f, $p<0.001$.

Variables	Men (n=48)			Women (n=52)		
	Baseline	At study end	Delta (%)	Baseline	At study end	Delta (%)
Age (yrs)	43.1±12.2[a]	-	-	38±12.8	-	-
BMI (kg/m^2)	44.7±4.2[f]	41.9±4	-6.2±1.9[c]	45.3±5.1[f]	43.1±4.8	-4.9±1.5
Waist (cm)	133.6±9.2[cf]	128±8.5[c]	-4.1±2.5	123.5±12.6[f]	117.9±11.9	-4.5±1.9
FPG (mg/dl)	104.5±32.5	-	-	94.4±19.9	-	-
FI (mIU/L)	25.5±40.7	-	-	19.1±15.5	-	-
HbA1c (%)	6.1±1.5	-	-	5.6±0.8	-	-
HOMA-IR	6.2±9.1	-	-	4.3±3.4	-	-
CRP (mg/dl)	0.8±0.9	-	-	1.03±0.8	-	-
CHO tot (mg/dl)	203.1±37.8	-	-	200.5±40.6	-	-
HDL CHO (mg/dl)	39.3±8.2	-	-	49.9±12.3	-	-
LDL CHO (mg/dl)	138.1±32.4	-	-	130.6±37.2	-	-
TG (mg/dl)	177.4±89.4	-	-	133±62.1	-	-
TSH (mIU/L)	2.12±0.36[e]	1.82±0.94	-10.5±29.9	2.06±0.89	1.91±0.89	-2.6±39.2
Free T3 (ng/L)	3.33±0.36[b]	3.26±0.28[c]	-1.6±9.1	3.12±0.33[e]	2.96±0.31	-4.8±10.2
Free T4 (ng/L)	11.96±1.92[f]	12.76±1.41[c]	8.3±14.8[b]	11.38±1.17	11.37±1.30	0.2±9.0
FT3/FT4 ratio	0.28±0.05[e]	0.26±0.03	-7.6±13.9	0.27±0.05	0.26±0.04	-3.9±12.8
REE (Kcal/24 h)	2247±337[ce]	2122±307[c]	-4.7±12.3	1841±298[e]	1768±233	-3.6±9.4
REE/pREE (%)	91.2±10.0[a]	88.2±10.9[b]	-0.12±0.5	96.7±11.3	95±8.5	-0.11±0.5
RQ (VO2/VCO2)	0.78±0.08	0.79±0.07	3.0±13.1	0.78±0.07	0.81±0.07	3.3±12.2
FM (%)	41.5±6.2[ce]	39.7±4.3[c]	-3.6±8.7[b]	51.3±3.1	51.4±3.1	0.1±3.3
FFM (kg)	77.9±9.7[ce]	75.5±8[c]	-2.4±10.5	57.5±6.5[f]	54.6±5.8	-4.9±3.5

For abbreviations: BMI, body mass index; FPG, fasting plasma glucose; FI, fasting insulin; HbA1c, glycated haemoglobin; HOMA-IR, homeostatic model of insulin resistance; CRP, C-reactive protein; CHO, cholesterol; HDL, high density lipoprotein; LDL, low-density lipoprotein; TG, Tryglicerides; REE, resting energy expenditure; pREE, predicted REE; REE/pREE, metabolic efficiency; RQ, respiratory quotient; FM, fat body mass; FFM, fat-free body mass.

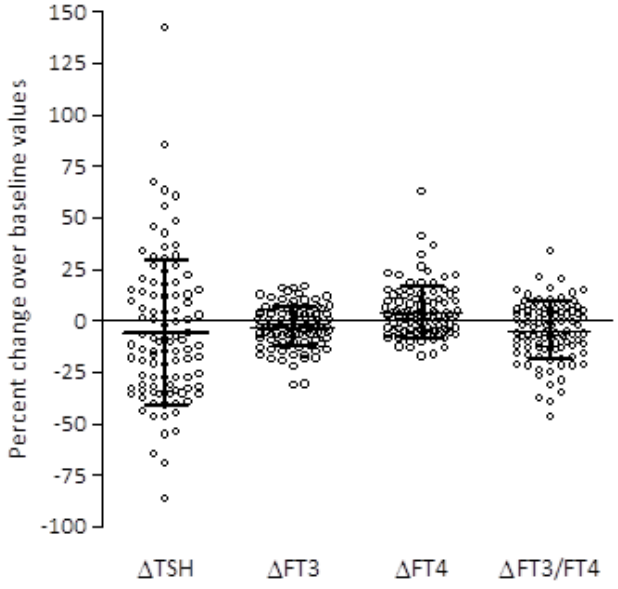
Table 3. Bivariate correlation analysis between main hormone and anthropometric study variables recorded at baseline and at the study end.

Variables	r	p
BMI (kg/m2)	0.984	0.0001
Waist (cm)	0.970	0.0001
TSH (mIU/L)	0.709	0.0001
FT3 (pg/L)	0.543	0.0001
FT4 (pg/L)	0.608	0.0001
FT3/FT4 ratio	0.521	0.0001
REE (kcal/24h)	0.791	0.0001
REE/pREE (%)	0.475	0.0001
RQ (VO2/VCO2)	0.189	0.06
FM (%)	0.879	0.0001
FFM (kg)	0.941	0.0001

For abbreviations: BMI, body mass index; REE, resting energy expenditure; pREE, predicted REE; REE/pREE, metabolic efficiency; RQ, respiratory quotient; FM, fat body mass; FFM, fat-free body mass.

Figures

Figure 1. Percent variations recorded at the study end over baseline values for TSH (ΔTSH), FT3 (ΔFT3), FT4 (ΔFT4), and the FT3/FT4 ratio (ΔFT3/FT4). Individual deltas, mean and standard deviations are displayed.



Obesity has complex effects on the metabolic outcome of hypothyroid patients on L-T4 therapy

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Abstract

Background. The general prevalence of inadequate L-T4 replacement therapy approaches 40%. Obesity carries a high disease burden, and biochemical control of hypothyroidism is essential to avoid the harmful health consequences of suboptimal thyroid hormone replacement. There is no information on obesity-related metabolic homeostasis in relation to L-T4 therapy and its adequacy.

Methods. Of 4950 obese patients, 690 hypothyroid subjects were selected as treated with L-T4 (L-T4 Group) and 773 euthyroid subjects were included as matched controls (Eu-Thyr Group). Analysis included TSH and fT4 levels, gluco-lipid homeostasis, fat-free mass (FFM) and percent fat mass (%FM), resting energy expenditure (REE).

Results. Inadequate replacement was observed in 171 patients (24.8%), comprising 7.5% over-replaced and 17.2% under-replaced. Compared to adequately treated patients, over-replaced patients took a higher L-T4 dose/kg/die, were younger and had lower BMI ($p=0.001$ for all) and waist circumference (WC) ($p<0.05$), whereas under-replaced patients took a lower L-T4 dose/kg/die ($p<0.05$), were younger ($p=0.001$), had higher FFM ($p<0.0001$), REE ($p<0.05$), WC ($p=0.01$), insulin levels ($p<0.05$) and HOMA-IR ($p=0.01$) and lower FM ($p=0.01$). Multinomial logistic regression analyses showed that under-replacement was associated with male gender ($p<0.05$), while over-replacement was associated with younger age ($p=0.001$), higher L-T4 dose/Kg/die ($p<0.01$) and lower BMI ($p=0.01$).

Conclusions. Obesity has complex effects on the metabolic outcome of hypothyroid patients in replacement therapy. However, there are potential confounders to consider for the management of hypothyroidism in obesity.

Background

Obesity and hypothyroidism are common clinical conditions linked together closely. The link has become more relevant in the context of an unprecedented rise in the prevalence of obesity worldwide *(Sanyal 2016)*.

Body composition and thyroid hormones appear to be closely related *(Marzullo 2016)*. In fact, thyroid hormones regulate basal metabolism, thermogenesis and play an important role in lipid and glucose metabolism, food intake and fat oxidation *(Rosenbaum 2000)*. Therefore, thyroid dysfunction is associated with changes in body weight and composition, body temperature and resting energy expenditure (REE) independent of physical activity. In particular, the alteration in body weight associated with hypothyroidism may reflect both the accumulation of body fat *(Seppel 1997, Wolf 1996)*, due to decreased REE, and the increased the body water content *(Smith 1989)*, consequent to a reduced capacity of excreting free water *(Skowsky 1978)*.

Replacement therapy with thyroid hormone is indicated as a treatment when the diagnosis of persistent thyroid hormone deficiency is confirmed *(Singer 1995, Surks 2004, Garber 2012)*. Levothyroxine (L-T4) is considered to be the treatment of choice in hypothyroid patients *(Roti 1993)*. The daily dosage of L-T4 is dependent on age, sex and body size *(Roti 1993, Woeber 2002)*. Ideal body weight is best used for clinical dose calculations because lean body mass is the best predictor of daily requirements *(Santini 2005)*. With little residual thyroid function, replacement therapy requires approximately from 1.6 μg/kg to 1.8 μg/kg of L-T4 daily *(Fish 1987, Devdhar 2011)*. Patients who are athyreotic after total thyroidectomy may require higher doses *(Kabadi 1995)*, while patients with subclinical hypothyroidism may require less *(Teixeira 2008, Meier 2001)*. Adequacy of L-T4 treatment is generally monitored by measurements of serum fT4 and TSH. Restoration of euthyroidism is followed by an increase in REE and even small variations in serum TSH, induced by L-T4 substitution, are associated with opposite changes in REE *(al-Adsani 1997, Boeving 2011, Santini 2014)*. However, in spite of adequate substitution with L-T4, hypothyroid patients may experience only a modest and/or transient loss of weight during hormone treatment *(Santini 2014, Karmisholt 2011)*. Excretion of excess body water, rather than reduction in fat mass, accounts for this change of body weight *(Santini 2014)*.

Although management of L-T4 therapy has been considered relatively straightforward, several studies in the past 2 decades have indicated aspects of previously unrecognized complexity despite the improvement in TSH and thyroid hormone assay *(Roti 1993, Biondi 2008, Cooper 2012)*. Epidemiological studies have reported that inadequate thyroid hormone replacement is present in over a third of L-T4-treated hypothyroid patients *(Canaris 2000, Somwaru 2009)* despite frequent biochemical monitoring *(Okosieme 2011, Liwanpo 2009)*.

In obesity, the prevalence of adequate substitution with L-T4 and its association with bio-anthropometric and metabolic parameters is, to date, unknown. This study was therefore designed to investigate the prevalence of adequate substitution with L-T4 therapy in obese patients, and to analyze its role in influencing body composition, REE and glucose-lipid metabolism.

Methods

Patients

Of 4950 obese patients admitted to our center between 2011-2014, this study enrolled 1463 patients, consisting of 690 hypothyroid obese subjects treated with tablet L-T4 (L-T4 Group) [605F/86M, age: 59 (IQR 50-69) years, BMI 43.9 (IQR 40.1-48.4) Kg/m2] and 773 euthyroid obese subjects (Eu-Thyr Group) [667F/106 M, age: 56 (IQR 48-65) years, BMI 43.5 (IQR 39.8-48.2) Kg/m2], referred to our institution for work-up and rehabilitation of obesity and its comorbidities. Written consent was obtained from all patient, after full explanation of the purpose and nature of the study. The investigation was approved by the local ethical committee, functioning according to the 3rd edition of the Guidelines on the Practice of Ethical Committees in Medical Research. Exclusion criteria included age below 18 or above 85 years, previous or current use of medications potentially interfering with thyroid function (such as amiodarone, steroids or lithium carbonate therapy), pregnancy, liver or kidney disease. Screening test included thyroid function tests (TSH, fT4), lipid profile, glucose and insulin levels, in fasting conditions. Patients were not prescribed diet therapy, dietary supplements, or antiobesity compounds for at least 3 months prior to entering the study.

Body measurements

All subjects underwent body measurements wearing light underwear, in fasting conditions after voiding. Weight and height were measured to the nearest 0.1 kg and 0.1 cm, respectively. BMI was expressed as body mass (kg)/height $(m)^2$. Obesity was defined for any BMI over 30 kg/m^2. Waist circumference (WC) was measured midway between the lowest rib and the top of the iliac crest after gentle expiration; hip was measured as the greatest circumference around the nates. Anthropometric data were expressed as the mean of two measurements.

Fat mass (FM) expressed as percentage of total body mass and free fat mass (FFM) expressed in kilograms, were assessed by bio-impedance analysis (BIA, 101/S Akern; Florence, Italy) the morning after overnight fasting and after voiding. The two vector components of impedance (i.e. resistance and reactance) were obtained by single measurements; before each testing session, the external calibration of the instrument was checked with a calibration circuit of known impedance value. The mean coefficient of variation was 1% for within-day and 3% for weekly intraindividual measurements in the steady-state condition in either site and 2% for interoperator variability.

Resting energy expenditure (REE) was expressed in kilocalories per 24 h an determined in a thermoregulated room (22-24°C) by computed open-circuit indirect calorimetry, measuring resting oxygen uptake and resting carbon dioxide production by a ventilated canopy (Sensormedics, Milan, Italy) at 1-min intervals for 30 min and expressed as 24 h value.

Laboratory test

Undiluted serum samples were assayed for fT4 and TSH using an automated chemiluminescence assay system (Immulite 2000; DPC, Los Angeles, CA). The principle of the method is a two-site, solid-phase chemiluminescent immunometric assay or competitive immunoassay. Normal values for TSH are 0.27-4.20 mU/L, and for fT4 9.0-17.0 ng/L.

Routine laboratory data including glucose, total cholesterol (CHO), high-density (HDL) and low-density lipoprotein (LDL) cholesterol and triglycerides (TG) were measured by

enzymatic methods (Roche Diagnostics, Mannheim, Germany). Insulin levels were measured using a Cobas Integra 800 Autoanalyzer (Roche Diagnostics, Indianapolis, IN, USA).

Insulin resistance was calculated by the homeostatic model of insulin resistance (HOMA-IR) index: insulin (mIU/L) × [glucose (mmol/L)/22.5] *(Matthews 1985)*. A HOMA-IR value greater than 2.0 was considered indicative of insulin resistance, as obtained in a sample of the Italian population *(Bonora 2000)*. The homeostatic model of β cell function (HOMA-B) was used to describe the functionality of pancreatic beta cells and calculated using the following formula: 20 [insulin (mIU/L)/glucose (mmol/L) − 3.5] *(Matthews 1985)*.

Statistics

Statistical analysis were performed using SPSS version 21 (Somers, NY, USA) on log transformed data to correct for the non-Gaussian distribution obtained by the Shapiro-Wilk test. Values are expressed as median and interquartile ranges. Univariate Anova test was used for comparison between groups. Spearman's correlation analyses were used to identify significant associations between variables of interest. Stepwise multivariate regression analysis was used to evaluate the independent association of variations in FM and FFM with metabolic, anthropometric or biochemical parameters. The multilinear model included age, gender, L-T4 dose, TSH and fT4 levels as independent variables. β coefficients and related significance values obtained from the models are reported. Multinomial logistic regression analyses were performed to test the effect of under- and over-replacement L-T4 therapy and other variables of interest on the metabolic parameters. Statistical significance was set at 5%.

Results

A summary of anthropometric and >40 kg/m^2 in 75.2% (1100 patients), >35.0-39.9 kg/m2 in 18.4 % (269 patients), and >30.0-34.9 kg/m^2 in 6.4% (94 patients).

Anthropometric and metabolic parameters differed between groups (Table 1, pag. 234). LT4 Group exhibited lower FFM ($p<0.01$) and higher %FM ($p<0.0001$) than Eu-Thyr Group. Nevertheless, cholesterol levels were lower in the former. Excluding over- and under-

treated patients from the L-T4 Group, we substantially found the same differences between the two groups as regard anthropometric and biochemical parameters.

L-T4 Group showed higher TSH and fT4 levels than Eu-Thyr Group. Inadequate replacement therapy was observed in 171 patients (24.8%), comprising 52 over-replaced (7.5%) and 117 under-replaced (17.2%). Compared to adequately treated patients, over-treated patients took a higher L-T4 dose/kg/die, were younger and had lower BMI ($p=0.001$ for all) and WC ($p<0.05$) (Table 2, pag. 235), whereas under-replaced patients took a lower L-T4 dose/kg/die ($p<0.05$), were younger ($p=0.001$), had higher FFM ($p<0.0001$), REE ($p<0.05$), WC ($p=0.01$), insulin levels ($p<0.05$) and HOMA-IR ($p=0.01$) and lower FM ($p=0.01$) (Table 3, pag. 236).

Analysis of thyroid function in Eu-Thyr Group showed a mild increase in TSH levels in a subset of 30 patients (3.9%). None of these harbored any increase in anti-thyroid antibody titer or abnormal free thyroid hormone levels.

Correlation analysis in the L-T4 Group showed opposite relationships between TSH and L-T4 dose/kg/die (rho=-0.20, $p<0.0001$), fT4 levels (rho=-0.34, $p<0.0001$) autoimmune origin of hypothyroidism (rho=-0.103, $p<0.01$), and positive relationships between TSH and BMI (rho=0.13, $p=0.001$), WC (rho=0.15, $p<0.0001$), FFM (rho=0.20, $p<0.0001$) and REE (rho=0.12, $p<0.0001$). These correlations remained after controlling for age, gender and smoking habit. Inversely, fT4 was opposely associated with BMI (rho=-0.08, $p<0.05$), WC (rho=-0.09, $p<0.05$), CHO (rho=-0.09, $p=0.01$), LDL (rho=-0.09, $p<0.05$), TG (rho=-0.11, $p<0.01$), insulin levels (rho=-0.11, $p<0.01$) and FFM (rho=-0.10, $p<0.01$), and positively associate with L-T4 dose/kg/die (rho=0.29, $p<0.0001$). Except for correlation with L-T4 dose and TG levels, other correlations were lost after controlling for potential confounders.

Stepwise multivariate regression analyses performed on the LT4 Group, documented that FM was independently predicted by LT-4 dose/kg/die (standardized $\beta=-0.13$, $p<0.0001$) as well as by female gender (standardized $\beta=-0.54$, $p<0.0001$), whereas FFM was independently predicted by LT-4 dose/kg/die (standardized $\beta=-0.08$, $p<0.01$) and TSH levels (standardized $\beta=-0.11$, $p<0.0001$) as well as by male gender (standardized $\beta=0.56$, $p<0.0001$) and age (standardized $\beta=-0.26$, $p<0.0001$).

However, when we performed this analysis on the whole population, we found that FM was independently predicted by the condition of hypothyroidism (standardized $\beta=-0.114$,

p<0.0001), female gender (standardized β=-0.11, p<0.0001) and age (standardized β=0.063, p<0.01), whereas FFM was independently predicted by the condition of hypothyroidism (standardized β=-0.12, p<0.0001), male gender (standardized β=-0.51, p<0.0001) and age (standardized β=0.06, p<0.01).

Multinomial logistic regression analyses (Table 4, pag. 237) showed that under-replacement was associated with male gender (OR 2.373, 95% CI 1.101-5.111, p<0.05), while over-replacement was associated with younger age (OR 0.963, 95% CI 0.941-0.986, p=0.001), higher L-T4 dose/Kg/die (OR 2.976, 95% CI 1.442-6.144, p<0.01) and lower BMI (OR 0.930, 0.878-0.985, p=0.01).

Discussion

Most of the studies evaluating appropriate thyroid replacement in hypothyroid patients were performed on normal weight or overweight individuals *(Okosieme 2011, Glymph 2016)*. The recommended dose of L-T4 currently used has been largely studied and validated in non-obese subjects *(Fish 1987, Roos 2005, Jonklaas 2010)* and there is a lack of clinical evidence demonstrating safe and effective thyroid hormone replacement strategies in obese patients *(Glymph 2016)*. The therapeutic goal in hypothyroidism is to achieve patients' well-being and restore serum TSH levels within the reference range *(Vaidya 2008)*. However, inadequate replacement is common in patients receiving L-T4. Cross-sectional studies demonstrated that 30-50% of patients treated with levothyroxine remain biochemically under-treated or over-treated *(Canaris 2000, Somwaru 2009, Okosieme 2011, Hollowell 2002, Hannemann 2010)*. Our results showed that hypothyroidism in severe obesity seem to be associated with a better adequate replacement with lower L-T4 doses compared to normal weight patients. Moreover, L-T4 daily requirement was able to negatively predict both FFM and FM. In agreement with our results, Glymph et al demonstrated that obese hypothyroid females tended to require a lower L-T4 dose per kg compared with non-obese females *(Glymph 2016)*. However, these findings contrast with results reported by Santini et al, who demonstrated that excessive fat accumulation would imply higher doses of L-T4 to attain the same TSH reduction as in lean subjects *(Santini 2005)*. It is likely that the variations in their study design and ours, such as therapy with TSH-suppressive doses of L-T4, could explain the

contrasting results. Lean body mass has been found to be an important determinant of body weight in normal weight and obese subjects *(Wesche 1998)*. Therefore, the requirements for T4, either secreted by the thyroid or therapeutically administered, may depend on lean body mass *(Santini 2005)*. According to the role of lean mass in determining L-T4 disposal, the absolute value of L-T4 requirement was higher in males than in females, as a consequence of gender-related differences in body weight and body composition. Confirming previous studies *(Cunningham 1984)*, the well-known reduction of L-T4 needs associated to advancing age appeared dependent on a relative decrease in lean body mass. As regards FM, Ortega et al hypothesized that the increased levels of iodothyronine deiodinase 1 (D1) in adipose tissue of obese subjects could reflect the stimulation of this enzyme by locally formed leptin, and that, in turn, enhanced production of T3 involved in adipose tissue metabolism *(Ortega 2012)*. Although the lower L-T4 doses required in obesity than normal weight, could be justified by an increase in T3 production formed by D1, further studies are required to clarify this issue *(Ortega 2012)*.

Despite most of the hypothyroid patients were biochemically adequately replaced with L-T4, we observed higher %FM and lower FFM than euthyroid subjects. Different studies demonstrated that hypothyroid patients may experience only a modest reduction in fat mass *(Santini 2014)* and about 15% of subjects treated with L-T4 do not reach clinical euthyroidism *(Biondi 2014)* and continue to have a metabolic impairment. One hypothesis to explain this phenomenon could be that in many tissue, intracellular T3 levels cannot be predicted based on circulating thyroid hormone levels due to the actions of the types 2 and 3 deiodinases. Thus, in some tissues, the relatively higher serum T4 levels could impair intracellular T3 production via down-regulation of a deiodinase pathway *(Werneck de Castro 2015)*. On these bases, several studies evaluated the effect of combination treatment with L-T4 and L-T3 vs L-T4 alone *(Grozinsky-Glasberg 2006, Ma 2009)*. However, the benefits of combination therapy are still unproven *(Biondi 2016)*.

Hypothyroidism is often associated with lipid abnormalities, especially elevated total and LDL-cholesterol *(Cappola 2003)*. However, hypothyroid patients treated with L-T4 showed lower levels of total and LDL-cholesterol than euthyroid subjects. In agreement with our results, a recent meta-analysis demonstrated an overall beneficial effect of L-T4 substitution in improving total and LDL cholesterol *(Li 2017)*. Moreover, we confirmed

that no significant effect of L-T4 seems to exist in terms of serum HDL cholesterol or TG concentrations also in obese subjects *(Biondi 2008, Ito 2007, Efstathiadou 2001, Tagami 2010)*.

As far as glucose homeostasis is concerned, the few available studies showed no association with subclinical hypothyroidism *(Al Sayed 2006, Kowalska 2011, Brenta 2007)*, although different populations were studied. However, our result demonstrated that L-T4 substitution may reduce basal insulin levels, improving insulin sensitivity. These results were in agreement with those reported in the literature *(Velija-Asimi 2007, Bilic-Komarica 2012)*.

This study has several limitations including its retrospective nature, the lack of informations about the patients' history L-T4 administration compliance and fT3 assay. Moreover, we were unable to determine the source of generic L-T4 preparations, which could have affected thyroid hormone bioavailability, and we could not confirm the daily schedule of L-T4 administration in each subjects. Overall, the study results reflect a real-world approach to managing hypothyroidism in obese subjects.

Conclusions

In conclusion, obesity has complex effects on the metabolic outcome of hypothyroid patients in replacement therapy. Our study demonstrated that severe obese patients required lower L-T4 doses compared to normal weight subjects. However, there are potential confounders to consider for the management of hypothyroidism in obesity. Further studies are needed to investigate the effects of obesity in the metabolic setting of hypothyroidism.

Tables

Table 1. Summary of anthropometric and biochemical data obtained in the LT4 Group and the Eu-Thyr Group.

Variables	LT4 group (n = 690)	Eu-Thyr group (n = 773)	p
Males/females	86/605	106/667	0.64
Age (years)	**59 (50-69)**	**56 (48-65)**	**<0.000**
BMI (kg/m^2)	43.9 (40.1-48.4)	43.5 (39.8-48.2)	0.529
Waist (cm)	123 (114-132)	122 (111-131)	0.067
REE (kcal/day)	1731 (1557-1940)	1733 (1515-2000)	0.901
FM (%)	**50.9 (46.6-54.2)**	**48.8 (44.0-52.9)**	**<0.000**
FFM (Kg)	**54.0 (48.3-59.8)**	**55.0 (49.6-63.3)**	**<0.01**
TSH (mU/L)	**2.08 (0.96-3.69)**	**1.80 (1.23-2.73)**	**<0.05**
fT4 (ng/L)	**12.1 (10.8-13.6)**	**11.2 (10.2-12.6)**	**<0.000**
Glucose (mg/dL)	97.0 (88.0-114.8)	95.0 (85.0-110.0)	0.123
Insulin (mU/L)	**13.1 (8.9-18.4)**	**13.7 (9.9-19.5)**	**<0.01**
HOMA-IR	3.2 (2.1-4.8)	3.3 (2.2-5.2)	0.059
HOMA-B	**136.0 (78.4-209.7)**	**154.8 (97.0-250.4)**	**<0.000**
CHO (mg/dL)	**191 (166-219)**	**202 (180-231)**	**<0.000**
LDL-CHO (mg/dL)	**120 (97-144)**	**128 (104-152)**	**<0.000**
HDL-CHO (mg/dL)	47 (39-56)	47 (39-54)	0.774
TG (mg/dL)	130 (100-175)	129 (102-169)	0.533

BMI, body mass index; WHR, Waist-to-hip ratio, REE, resting energy expenditure; FM, fat mass; FFM, free fat mass; TSH, thyroid-stimulating hormone; fT4, free thyroxine; HOMA-IR, homeostatic model of insulin resistance; HOMA-B, homeostatic model of β cell function; CHO, total cholesterol; LDL, low density lipoprotein; HDL, high density lipoprotein; TG, triglycerides.

Table 2. Summary of anthropometric and biochemical data obtained in the adequately treated and over-replaced patients.

Variables	Adequately treated (n = 519)	Over-replaced (n = 52)	p
Males/females	50/469	6/46	0.660
Age (years)	**60 (51-70)**	**54 (48-63)**	**0.001**
Dose (µg/kg/die)	**0.900 (0.617-1.136)**	**1.157 (0.938-1.371)**	**0.001**
BMI (kg/m^2)	**44.1 (40.2-48.5)**	**40.6 (37.1-43.7)**	**0.001**
Waist (cm)	**123 (113-132)**	**120 (110-125)**	**0.021**
REE (kcal/day)	1720 (1549-1920)	1711 (1570-1904)	0.976
FM (%)	51.0 (47.2-54.5)	50.0 (44.7-53.0)	0.113
FFM (Kg)	53.7 (47.9-58.8)	51.1 (47.7-56.8)	0.217
TSH (mU/L)	**1.80 (1.02-2.80)**	**0.07 (0.03-0.15)**	**<0.000**
fT4 (ng/L)	**12.1 (11.0-13.6)**	**14.3 (12.7-16.0)**	**<0.000**
Glucose (mg/dL)	96 (88-113)	95 (88-107)	0.401
Insulin (mU/L)	12.7 (8.9-17.7)	13.8 (9.6-18.3)	0.202
HOMA-IR	3.0 (2.1-4.6)	3.2 (2.2-5.0)	0.374
HOMA-B	131.8 (78.2-205.1)	155.3 (96.8-234.9)	0.064
CHO (mg/dL)	192 (168-218)	181 (155-213)	0.208
LDL-CHO (mg/dL)	120 (99-143)	104 (89-140)	0.283
HDL-CHO (mg/dL)	47 (38-57)	47 (41-53)	0.926
TG (mg/dL)	127 (102-174)	126 (85-153)	0.278

BMI, body mass index; WHR, Waist-to-hip ratio, REE, resting energy expenditure; FM, fat mass; FFM, free fat mass; TSH, thyroid-stimulating hormone; fT4, free thyroxine; HOMA-IR, homeostatic model of insulin resistance; HOMA-B, homeostatic model of β cell function; CHO, total cholesterol; LDL, low density lipoprotein; HDL, high density lipoprotein; TG, triglycerides.

Table 3. Summary of anthropometric and biochemical data obtained in the adequately treated and under-replaced patients.

Variables	Adequately treated (n = 519)	Under-replaced (n = 119)	p
Males/females	**50/469**	**30/89**	**<0.000**
Age (years)	**60 (51-70)**	**57 (49-64)**	**0.029**
Dose (µg/kg/die)	**0.900 (0.617-1.136)**	**0.841 (0.497-1.139)**	**0.024**
BMI (kg/m^2)	44.1 (40.2-48.5)	44.5 (41.0-49.6)	0.105
Waist (cm)	**123 (113-132)**	**127 (116-139)**	**0.012**
REE (kcal/day)	**1720 (1549-1920)**	**1809 (1631-2057)**	**0.029**
FM (%)	**51.0 (47.2-54.5)**	**49.5 (43.6-53.9)**	**0.012**
FFM (Kg)	**53.7 (47.9-58.8)**	**58.6 (50.9-68.4)**	**<0.000**
TSH (mU/L)	**1.80 (1.02-2.80)**	**5.90 (4.92-8.12)**	**<0.000**
fT4 (ng/L)	**12.1 (11.0-13.6)**	**11.1 (9.9-12.4)**	**<0.000**
Glucose (mg/dL)	96 (88-113)	101 (90-119)	0.175
Insulin (mU/L)	**12.7 (8.9-17.7)**	**14.3 (8.8-21.8)**	**0.030**
HOMA-IR	**3.0 (2.1-4.6)**	**3.6 (2.1-6.0)**	**0.011**
HOMA-B	131.8 (78.2-205.1)	136.8 (77.5-187.3)	0.373
CHO (mg/dL)	192 (168-218)	189 (166-226)	0.856
LDL-CHO (mg/dL)	120 (99-143)	121 (95-147)	0.629
HDL-CHO (mg/dL)	47 (38-57)	46 (40-55)	0.672
TG (mg/dL)	127 (102-174)	141 (100-183)	0.321

BMI, body mass index; WHR, Waist-to-hip ratio, REE, resting energy expenditure; FM, fat mass; FFM, free fat mass; TSH, thyroid-stimulating hormone; fT4, free thyroxine; HOMA-IR, homeostatic model of insulin resistance; HOMA-B, homeostatic model of β cell function; CHO, total cholesterol; LDL, low density lipoprotein; HDL, high density lipoprotein; TG, triglycerides.

Table 4. Multinomial adjusted Odds Ratios for High TSH level versus Nomal TSH level (PANEL A) and for Low TSH level versus Nomal TSH level (PANEL B).

	PANEL A		PANEL B	
Covariates	**TSH level: High vs Normal**		**TSH level: Low vs Normal**	
	OR (95% CI)	P-value	OR (95% CI)	P-value
Age	0.991 (0.974-1.007)	0.258	**0.963 (0.941-0.986)**	**0.001**
Gender				
Female	1	Ref.	1	Ref.
Male	**2.373 (1.101-5.111)**	**0.027**	1.746 (0.545-5.593)	0.348
BMI	1.012 (0.978-1.047)	0.507	**0.930 (0.878-0.985)**	**0.013**
Dose	1.023 (0.588-1.780)	0.935	**2.976 (1.442-6.144)**	**0.003**
Duration of disease	0.989 (0.958-1.021)	0.488	0.995 (0.952-1.039)	0.819
Fat free mass	1.012 (0.989-1.036)	0.300	0.981 (0.947-1.016)	0.284
Cause of disease				
- Autoimmune (0)	1	Ref.	1	Ref.
- Thyroidectomy (1)	0.562 (0.294-1.270)	0.166	1.375 (0.582-3.250)	0.468

CHAPTER 4

Other research interests

PITUITARY DYSFUNCTION:
HYPOPITUITARISM AND ACROMEGALY

Introduction

Hypopituitarism is deficiency of one or more pituitary hormones. The pituitary gland has two lobes. Within the anterior lobe, six hormones are produced: growth hormone, the gonadotropins follicle stimulating hormone (FSH) and luteinising hormone, adrenocorticotropic hormone (ACTH), thyroid stimulating hormone (TSH), and prolactin *(Higham 2016)*. The posterior pituitary lobe contains two hormones, oxytocin and antidiuretic hormone (ADH), which are produced in the supraoptic and paraventricular nuclei of the hypothalamus and transported axonally via the pituitary stalk to be stored and released from the posterior lobe *(Higham 2016)*.

In adults, the most common cause of hypopituitarism is a pituitary adenoma, treatment with pituitary surgery or radiotherapy, trauma and vascular injury (Tanriverdi 2014).

The profile of pituitary hormone deficiencies varies depending on the site within the hypothalamic–pituitary axis and the nature of the underlying pathological process *(Higham 2016)*. Hypopituitarism is diagnosed based on baseline blood sampling for thyroid stimulating hormone, gonadotropin, and prolactin defi ciencies, whereas for ACTH, growth hormone, and antidiuretic hormone deficiency dynamic stimulation tests are usually needed *(Karamouzis 2016)*.

Replacement treatment exists in the form of thyroxine, hydrocortisone, sex steroids, growth hormone, and desmopressin *(Higham 2016)*.

Acromegaly is a rare and underdiagnosed disease that results from the overproduction of growth hormone (GH) and insulin-like growth factor 1 (IGF-1) *(Melmed 2006)*. More than 95% of patients with acromegaly harbor a GH-secreting pituitary adenoma (somatotropinoma) arising from somatotroph cells, leading to GH and IGF-1 hypersecretion *(Katznelson 2014)*. In less than 5% of cases, excess growth hormone releasing hormone (GHRH) secretion from a hypothalamic tumor or a neuroendocrine tumor (usually from lung or pancreas origin) may lead to somatotroph hyperplasia and acromegaly *(Borson-Chazot 2012)*.

Over 90–95% of GH-secreting pituitary adenomas are sporadic and occur as an isolated disorder *(Melmed 2006)*. However, acromegaly can also occur in a familial setting, either associated with other endocrine abnormalities or as an isolated disorder *(Rostomyan 2015, Capatina 2015)*.

Acromegaly affects both males and females equally and the average age at diagnosis ranges from 40 to 50 years *(Holdaway 1999, Etxabe 1993)*. The clinical features of acromegaly are due to local effects of an expanding pituitary mass, as well as to the direct and indirect effects of excessive secretion of GH and IGF-1, which can lead to systemic complications and impaired quality of life (QoL) *(Melmed 2016, Molitch 1992)*. These clinical manifestations range from subtle signs of acral overgrowth, soft-tissue swelling, arthralgias, jaw prognathism, mild hyperglycemia, menstrual disturbances, erectile dysfunction and hyperhidrosis to facial and skeletal disfigurement, florid osteoarthritis, severe headache, sleep apnea, severe hypertension, diabetic ketoacidosis, and respiratory and cardiac failure *(Melmed 2016, Molitch 1992)*

This section aims to explore the clinical-diagnostic approach to patients with hypopituitarism due to trauma or vascular injury and to estimate prevalence and incidence of acromegaly in the Piedmont region, Italy.

Clinical and diagnostic approach to patients with hypopituitarism due to traumatic brain injury (TBI), subarachnoid hemorrhage (SAH), and ischemic stroke (IS)

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Abstract

The hypothalamic-pituitary dysfunction attributable to traumatic brain injury (TBI), aneurysmal subarachnoid hemorrhage (SAH), and ischemic stroke (IS) has been lately highlighted. The diagnosis of TBI-induced-hypopituitarism, defined as a deficient secretion of one or more pituitary hormones, is made similarly to the diagnosis of classical hypopituitarism because of hypothalamic/pituitary diseases. Hypopituitarism is believed to contribute to TBI-associated morbidity and to functional and cognitive final outcome, and quality-of-life impairment. Each pituitary hormone must be tested separately, since there is a variable pattern of hormone deficiency among patients with TBI-induced-hypopituitarism. Similarly, the SAH and IS may lead to pituitary dysfunction although the literature in this field is limited. The drive to diagnose hypopituitarism is the suspect that the secretion of one/more pituitary hormone may be subnormal. This suspicion can be based upon the knowledge that the patient has an appropriate clinical context in which hypopituitarism can be present, or a symptom known as caused by hypopituitarism. Hypopituitarism should be diagnosed as a combination of low peripheral and inappropriately normal/low pituitary hormones although their basal evaluation may be not distinctive due to pulsatile, circadian, or situational secretion of some hormones. Evaluation of the somatotroph and corticotroph axes require dynamic stimulation test (ITT for both axes, GHRH + arginine test for somatotroph axis) in order to clearly separate normal from deficient responses.

Background

Traumatic brain injury (TBI) is defined as an alteration in brain function, caused by an external force, being a major public health problem in nowadays *(Tanriverdi 2015)*. Neuroendocrine dysfunction following TBI may occur with a much higher prevalence than previously suspected *(Tanriverdi 2015)*. Similarly, subarachnoid hemorrhage (SAH) and ischemic stroke (IS) may lead to pituitary dysfunction although the literature in this field has largely developed lately as survival rates mainly from SAH have improved, enabling pituitary evaluation to be performed on large cohorts of long-term SAH survivors *(Hannon 2011, Bondanelli 2006)*.

Pituitary disorders are a frequently overlooked complication of TBI and SAH. Many symptoms of TBI and SAH survivors such as fatigue, concentration difficulties, depressive symptoms are nonspecific and overlap with symptoms of patients with pituitary disorders from other causes with a large amount of hypopituitarism related to brain damage remaining undiagnosed *(Hannon 2011, Tanriverdi 2011)*. This can explain why the diagnosis of hypopituitarism is often missed or delayed after these conditions with potentially serious and sometimes life-threatening consequences for the affected patients. Therefore, these patients should undergo routine screening for hypopituitarism and each pituitary hormone must be evaluated separately, since there is a variable pattern of hormone deficiency among patients with TBI-induced hypopituitarism *(Tanriverdi 2015)*.

Epidemiology and pathophysiology

The drive to diagnose hypopituitarism is the suspect that the secretion of one or more pituitary hormone may be subnormal. This suspicion has an appropriate clinical context in which hypopituitarism can be present, or symptoms known as caused by hypopituitarism *(Tanriverdi 2015)*. The knowledge that the patient had a TBI or SAH is robust enough to evaluate for hypopituitarism because some patients with hypopituitarism have no symptoms *(Tanriverdi 2015, Nannon 2011, Bondanelli 2006)*. On the other hand, considering the high number of subjects with TBI, it is crucial to strictly define on a

cost/benefit basis which patients should be investigated for hypopituitarism *(Tanriverdi 2011)*. Evidence shows that pituitary function is impaired at least in 20–30% of patients following moderate-to-severe TBI; thus, patients with clinical signs or symptoms associated with hypopituitarism should be screened for their pituitary function *(Ghigo 2005)*.

There is a wide variation in the frequencies of hormone deficits following TBI and SAH *(Tanriverdi 2015, Nannon 2011, Bondanelli 2006)*. Reported prevalence of TBI-induced hypopituitarism considerably differs among reported studies ranging from 5.4 to 90% *(Kokshoorn 2010)*. Nevertheless, approximately one-third of TBI patients will develop at least one anterior pituitary disorder *(Lauzier 2014)*. In particular, a meta-analysis including patients with TBI reported a prevalence of anterior hypopituitarism from 15 to 68% and a posterior endocrine dysfunction about 6.9% *(Schneider 2007)*. On the other hand, the evidences for pituitary dysfunction following SAH and IS are less respect to TBI *(Schneider 2007, Fernandez-Rodriguez 2015, Bondanelli 2010)*. Among long-term SAH survivors, the reported prevalence of anterior and posterior dysfunction was from 37.5 to 55% and 0 to 2.8%, respectively *(Schneider 2007)*. Similarly, hypopituitarism was detected from 23.5 to 37.5% in stroke patients *(Bondanelli 2006, Bondanelli 2010)*. These variabilities could be attributed to different timings of evaluation, severity of the trauma or stroke, diagnostic criteria, methods and assays to test endocrine function *(Lauzier 2014)*. Thus, the timing of hormonal assessment of the pituitary disorders at different time frames varied highly across studies *(Tanriverdi 2015)*. The transient hypopituitarism has been almost exclusively reported in the first 6 months after TBI as hormone alterations mimicking pituitary insufficiency can be present in the acute phase after trauma *(Kokshoorn 2010)*. An early assessment of pituitary function after the event could lead to overestimate prevalence of pituitary dysfunction in these patients, especially if patients with a more severe trauma are included. Indeed, the chance to develop hypopituitarism is directly related to the severity of trauma. A rational approach to endocrine testing could be with an assessment of HPA axis reserve in the acute phase followed by a more comprehensive assessment of pituitary function during the chronic recovery phase. Moreover, physiologic hormonal changes that can mimic pituitary dysfunction are often observed in the early post-traumatic period. The physiologic response to acute and critical illness comprises hormonal changes similar for GH deficiency, central hypogonadism, and

hypothyroidism. Furthermore, the metabolism of the protein-binding hormones can be altered by acute illness or drugs frequently used in severe diseases, resulting in increased circulating levels and, consequently, false deficiencies *(Fernandez-Rodriguez 2015)*.

The reliability of the methodological tools used to assess pituitary function could be another important factor *(Tanriverdi 2015)*. Evaluation of the GH and HPA axes requires dynamic stimulation test in order to clearly separate normal from deficient responses and normative cut-off should be defined using healthy volunteers in each local laboratory *(Tanriverdi 2015, Kelly 2000, Klose 2014)*. Therefore, differences in the reported frequency may be due to more stringent diagnostic criteria applied by some studies but not to others *(Tanriverdi 2015, Agha 2004, Kreitschmann-Andermahr 2004, Brandt 2004, Aimaretti 2004)*. Moreover, cut- off levels used for tests and possible different hormone assays might have another important influence on the frequency of hypopituitarism *(Tanriverdi 2015, Kelly 2000, Schneider 2006, Agha 2006)*. Therefore, the robustness of the methodologies used to diagnose hypopituitarism varies between studies but there is a broad agreement nevertheless that hypopituitarism is a common complication of TBI *(Tanriverdi 2015, Hannon 2011, Bondanelli 2006, Tanriverdi 2011, Ghigo 2005, Kokshoorn 2010, Lauzier 2014, Schneider 2007, Fernandez-Rodriguez 2015, Bondanelli 2010, Kelly 2000)*.

The pathophysiology of hypopituitarism following brain damage is not completely understood and several factors implicated in its development have been suggested. Different types of lesions could cause from damage to the pituitary capsule, to injury to the anterior and posterior lobe and pituitary stalk in the form of hemorrhage, necrosis, and fibrosis *(Schneider 2007, Richmond 2008)*. Moreover, vascular damage to the pituitary gland can also be caused by venous infarctions of the hypophyseal portal vessels or by pituitary stalk transaction *(Tanriverdi 2015, Schneider 2007)*. Finally, recent research has indicated a possible interaction between autoimmunity and the development of hypopituitarism after TBI. It has been demonstrated that antipituitary and antihypothalamic antibodies (Abs) are present in patients with TBI-induced pituitary dysfunction and persist even 5 years after diagnosis *(Tanriverdi 2008, Tanriverdi 2013)*. It seems that antibodies may form after TBI due to the disruption of the blood–brain barrier, which allows brain proteins to leak into the circulation, evoking an immune response *(Tanriverdi 2008)*. Moreover, in patients with higher titers of pituitary

antibodies, the development of pituitary deficiencies is more frequent whereas the recovery of pituitary function is related to negative antibodies titers *(Schneider 2011)*.

Several studies have highlighted the existing relationship between hypopituitarism and TBI-associated morbidity *(Tanriverdi 2015, Klose 2008)*. Serious and life-threatening adrenal crises secondary to acute ACTH hormone deficiency in patients with TBI have been highlighted in the literature with dramatic improvement following glucocorticoid replacement *(Agha 2005, Cohan 2005)*. Increased neuropsychiatric morbidity in patients with TBI with GH insufficiency also has been reported *(Kelly 2006)*. It is further expected that hypopituitarism significantly impedes recovery and rehabilitation after TBI *(Fernandez-Rodriguez 2015)*. In fact, posttraumatic hypopituitarism was independently associated with poor quality of life, abnormal body composition, and adverse metabolic profile *(Klose 2007)*. In patients with SAH, preliminary data indicate that neuroendocrine disturbances contribute to disturbed quality of life, depression, and sleeping disturbances *(Kreitschmann-Andermahr 2007)*. These findings indicate that hypopituitarism after both TBI and SAH is associated with poor recovery and worse outcome (Table 1, pag. 252).

Diagnostic process

Hypopituitarism should be diagnosed as a combination of low peripheral and inappropriately normal/low pituitary hormones. However, the measurement of basal hormones alone may be not distinctive due to pulsatile, circadian, or situational secretion of some hormones.

Hypothalamic–pituitary–adrenal (HPA) axis

The basal secretion of ACTH must be sufficient to maintain the serum cortisol concentration within the normal range. During physical stress, ACTH secretion must increase in order to raise serum cortisol concentrations adequately. Thus, normal cortisol levels do not exclude the ACTH inability to adequately respond to stress if impaired. ACTH and cortisol secretion follow a circadian rhythm with highest levels in the early morning hours and lowest levels around midnight. Thus, basal ACTH secretion, and serum cortisol should be measured at 8 to 9 AM.

In the appropriate clinical context, secondary adrenal insufficiency can be excluded at morning cortisol levels greater than 500 nmol/L (>18 mcg/dL) and is indicated below 83-100 nmol/L (<3 mcg/dL). Intermediate levels require a stimulation test *(Tanriverdi 2015, Arlt 2003, Lamberts 1998, Snyder 2007)*.

Hypoglycemia (blood glucose <2.2 mmol/L, 40 mg/dL) induced by the insulin tolerance test (ITT, 0.05, 0.1–0.2 IU insulin per kg body weight given iv as a bolus) is considered as the gold standard for assessment of the HPA axis. A cortisol peak level greater than 500 nmol/L (18 mcg/dL) indicates normal integrity of the axis. This test has some unpleasant side effects such as sweating, trembling, fatigue, and hunger, and is contraindicated in elderly patients and in those with cardiac, cerebrovascular or epileptic seizures. In the under-study population, this test has been used by some authors *(Kelly 2000, Agha 2004, Brandt 2004, Kreitschmann-Andermahr 2004)* without adverse effects, but other authors have used alternative tests, suggesting the possible danger of ITT *(Ghigo 2005, Aimaretti 2004)*. It should be conducted only under close supervision in experienced centers *(Tanriverdi 2015, Ghigo 2005, Schneider 2006, Landon 1966)*.

Corticotropin releasing hormone (CRH, 100 lg as a bolus), given as a stimulus for the pituitary ACTH reserve, has proven to be not more predictive of adrenal function than morning cortisol levels *(Tanriverdi 2015, Dullaart 1999)*. Therefore, it is of limited value for the diagnosis of ACTH deficiency.

Overnight metyrapone test (MET) has so far been considered a convenient and sensitive method, with a good correlation with ITT *(Fiad 1994)*. MET (30 mg/kg administered orally with a snack at midnight) inhibits adrenal 11b-hydroxylase and the conversion of 11-deoxycortisol (11-DOC) to cortisol with a possible risk of precipitating adrenal crisis. Shortcomings of the MET test are the limited availability of reliable 11-DOC assays and the fact that the drug is not widely available owing to local restrictions *(Fiad 1994)*.

ACTH deficiency causes adrenal atrophy and ACTH receptor down-regulation. This is the rationale for the administration of exogenous ACTH *(Tanriverdi 2015, Arlt 2003, Lamberts 1998)*. Thus, the 250 μg 1-24 ACTH test could be used to establish secondary adrenal insufficiency if performed at least 4 weeks after onset of ACTH deficiency *(Tanriverdi 2015, Arlt 2003, Snyder 2007)*. It is administered intramuscularly or intravenously measuring serum cortisol 30 and 60 min later. A serum cortisol concentration ≥500

nmol/L (>18 mcg/dL) is considered a normal response. Stimulated cortisol levels ≤500 nmol/L indicate ACTH deficiency *(Tanriverdi 2015, Snyder 2007, Lindholm 1987)*.

It has been proposed that the low-dose (1 μg) corticotrophin test represents a more physiological stimulus for maximal adrenal stimulation compared to the 250 μg *(Tanriverdi 2015, Arlt 2003, Snyder 2007)*. Some studies have suggested a superior sensitivity for the 1 μg corticotrophin test whereas a meta-analysis reported comparable diagnostic accuracy of both tests *(Agha 2006)*.

The low-dose test has technical disadvantages (dilution in particular), and the need for repetitive blood sampling. This makes the standard test more practical and easy performed. Moreover, in a retrospective study where 148 patients with a low-normal cortisol response to the 250 lg test were studied over a median time of 4.2 years; only two patients developed a clear-cut adrenal insufficiency, another two presented a persisting diagnostic uncertainty, and seven had adrenal insufficiency after subsequent pituitary surgery or irradiation *(Agha 2006)*. Thus, the 250 μg ACTH test seems an appropriate diagnostic tool to exclude clinically significant hypoadrenalism, even though, definite conclusion, including a comparison with the ITT, would be desirable.

In the mean time, glucagon stimulation test (GST, 1 mg glucagon s.c.) has been started to be used more frequently in the evaluation of the HPA axis *(Simsek 2015, Leong 2001)*. Blood samples for measurement of cortisol were obtained 90, 120, 150, 180, 210, and 240 min after glucagon injection. The cut-off cortisol level ≥500 nmol/L accepted as sufficient responses

to ITT seems to reveal a low specificity in GST *(Berg 2010)*. In different studies, it was demonstrated that the minimum peak cortisol response of an healthy individual could be as low as 200, 250, and 295 nmol to GST *(Karaca 2011, Tanriverdi 2007)* although the majority of healthy adults were demonstrated to have peak cortisol responses ≥500 nmol/L to GST *(Rao 1987)*. It is likely too high and will likely misclassify many healthy adults as being adrenally insufficient. Thereby, as suggested by Berg et al., a lower cutpoint is likely required in order to avoid many false positives with regard to cortisol status during the glucagon test with the threshold of 277 nmol/L being more specify and sensitive *(Berg 2010)*. The reproducibility of the GST seems to be about 88% in the evaluation of the HPA axis and GST may be a good alternative. In a recent study by Simsek et al., comparing ITT, low-dose ACTH test and GST with each other, it has been shown

that the peak cortisol responses to all three tests were found to be well correlated with each other. The concordance of all 3 tests was found to be only about 44–50% according to the cut-off level of 500 nmol whereas higher concordance was detected when new cutoff level for peak cortisol response were used. Consequently, any test can be used in the evaluation of the HPA axis, but cut-off levels for the insufficiency of HPA axis should be individualized for each test *(Simsek 2015)*. Additionally, it has to be borne in mind that no test, including the ITT, classifies all subjects correctly while even healthy individuals might show abnormal values *(Tanriverdi 2015, Arlt 2003, Mayenknecht 1998, Soule 2000, Nye 2001, Suliman 2002)*. Thus, in borderline cases, clinical judgment and careful follow-up is crucial in the evaluation of HPA axis.

TRH-TSH-thyroid axis

In the appropriate clinical context, central hypothyroidism is easily diagnosed when the levels of free T4 are decreased and TSH levels are low or more often normal. Dynamic testing is generally not necessary as it does not add to diagnostic reliability. T3 is still normal in most cases *(Ferretti 1999, Westwood 2000, Hartoft-Nielsen 2004, Mehta 2003)*.

GnRH-LH/FSH-gonadal axis

In both sexes. Before the diagnosis of GnRH-LH/FSH gonadal axis function, prolactin (PRL) excess should be excluded that may be present due to disturbed hypothalamic inhibition of prolactin release *(Tanriverdi 2015, Ghigo 2005, Lamberts 1998, Snyder 2007)*. Hypogonadism in pre-peripubertal children does not show specific clinical symptoms until the onset of puberty. Then it usually presents with delayed or missing onset of puberty *(Tanriverdi 2015, Mehta 2003, Schneider 2007)*.

In a woman of premenopausal age who has pituitary or hypothalamic disease like TBI/SAH but normal menses, no tests of LH or FSH secretion are needed because a normal menstrual cycle is a more sensitive indicator of intact pituitary–gonadal function than any biochemical test. If a-, oligo-menorrhoea is present with low estradiol levels and inappropriately low/normal LH and FSH levels secondary hypogonadism in premenopausal women is diagnosed. In peri-post menopause, low LH/FSH levels shows

central hypogonadism (Figure 1, pag. 253) *(Tanriverdi 2015, Ghigo 2005, Lamberts 1998, Snyder 2007)*.

In man, LH deficiency can best be detected by measurement of the serum testosterone concentration. If it is repeatedly low at 8 to 10 AM and the LH concentration is not elevated, the patient has secondary hypogonadism. When the serum testosterone concentration is low, the serum LH concentration is usually within the normal range, but low compared with elevated values in primary hypogonadism. If fertility is an issue, the sperm count should be determined *(Tanriverdi 2015, Ghigo 2005, Lamberts 1998, Snyder 2007)*.

Dynamic test, GnRH in particular, is generally not necessary in adults because it does not add greater diagnostic accuracy. GnRH test could be useful in adolescents when a differential diagnosis between hypogonadism and pubertal delay is cumbersome (*Tanriverdi 2015, Lamberts 1998, Snyder 2007)*.

Somatotroph axis

The somatotroph (GH-IGF-I) axis needs to be evaluated by stimulation test, unless all other pituitary axes are deficient and insulin-like growth factor-1 (IGF-1) is low *(Tanriverdi 2015, Casanueva 2005, Molitch 2011)*.

Some studies show that the evidence of low age-related IGF-I level could be diagnostic ‘‘per se’’ only if all other anterior pituitary hormone are already compromised *(Hartman 2002, Aimaretti 2003)*. Measurement of basal serum growth hormone concentration does not distinguish reliably between normal and subnormal growth hormone secretion in adults *(Tanriverdi 2015, Aimaretti 1998, 1999, 2003, GHRS 2000)*.

For the diagnosis of GH deficiency, the ITT and the GHRH+arginine test are now considered as the tests of choice with similar accuracy *(Aimaretti 2003)*.

For the ITT test a peak GH response levels lower than 3 µg/L indicate severe GH deficiency in adults (if hypoglycaemia is adequately reached) *(Tanriverdi 2015, Simsek 2015, Aimaretti 2003)*.

The GH releasing hormone plus arginine test (GHRH+ARG, 1 µg/kg GHRH iv as a bolus plus 30 g arginine as an infusion over 30 min) is easy to perform, well tolerated, and has been shown to reliably detect severe GH deficiency in a lean adult population when a cut-off of

9 µg/L is used *(Ghigo 1996, Aimaretti 1998)*. But, the GH response to GHRH+ARG, and to all known GH provocative stimuli, significantly declines with increasing body mass index in adults *(Bonert 2004, Qu 2005)*; thus the use of cut-off non BMI-related in obese subjects causes a high percentage of false positive results *(Arlt 2003)*. The study from Biller and coworkers, suggesting for the GHRH+ARG test a cut-off level of 4.2 µg/L is indicative for GH deficiency in obese subjects *(Biller 2002)*. Similarly our group *(Corneli 2005, 2007)* using the Roc-analysis defined the BMI-dependent cut-off levels both in adults and in transition populations. The cut-off levels suggested for lean (BMI<25), overweight (BMI ≥25–30), and obese (BMI ≥30) adults subjects are 11.5; 8.0, and 4.1 µg/L, respectively *(Corneli 2007)*. A GH peak below this limit is indicative of GH deficiency in the appropriate clinical context. Similar results, considering waist circumference instead of BMI was reached by Colao et al. in a collaborative recent study *(Colao 2009)*. Moreover, it is known that the response of pituitary GH release as stimulated by multiple individual secretagogues is decreased in aging and elevated thyroid hormone concentrations. However, somministration of arginine enhances GHRH's stimulatory effect on GH release in the elderly by fourfold, and evokes normal GH secretion acutely *(Giustina 1995, 1998)*.

Further, there is evidence in patients receiving chronic glucocorticoid treatment that arginine is able to normalize the GH response demonstrating that the stimulatory action of arginine and the inhibitory action of glucocorticoids on GH secretion are mediated by opposite effects on hypothalamic somatostatin tone *(Giustina 1992)*. Another potent and validated provocative test is the GHRH+GH releasing peptide-6 test *(Leong 2001, Aimaretti 1998)*. It has BMI-dependent cut-off (15 and 5 mcg/L for lean and obese with BMI >35 kg/m^2, respectively) and possess a great accuracy in distinguish normal subjects from patients with GH deficiency *(Popovic 2000, Kelestimur 2006)*.

Unfortunately GHRH is no longer available in the United States. Other classical stimuli such as arginine alone, clonidine, L-DOPA, and the combination of arginine and L-DOPA are much weaker and therefore more likely to give false positive results and useless in adults patients *(Aimaretti 2003)*. The glucagon test (GST) (1 mg glucagon im, GH measurements every 30 min until 240 min after administration) has been proposed as alternative because it is able to separate with a sensitivity and specifity of 100% between GH deficient and healthy subjects if a peak GH of 3 µg/L is considered *(Molitch 2011, Gomez 2002)*. GST is a good alternative when GHRH is unavailable or there is

contraindication to ITT However, like the other tests, it is age- and BMIdependent *(Giustina 1998)* and more time-consuming than other stimulation tests *(Hartoft-Nielsen 2004)* although revised cut-offs might be taken in consideration in the field of the diagnosis of GHD *(Diri 2015)*.

In all, for the diagnosis of adult GHD in TBI patients we need to consider that no test for the assessment of GH deficiency is totally reliable. The likelihood of GH deficiency in TBI patients increases with increasing numbers of additional pituitary axes deficiencies *(Gasco 2012)*.

Posterior pituitary

Diabetes Insipidus (ADH deficiency) causes polyuria and polydipsia. Diabetes mellitus as a common cause of polyuria should be excluded. Diabetes insipidus is likely if polyuria (>40 mL/kg body weight/day, >2500–3000 cc day) in combination with urine osmolality <300 mOsm/kg and hypernatriaemia is present. It can be clearly manifested in the acute phase and requires treatment acutely. In case of normal plasma sodium levels, a water deprivation test will be necessary but it should be effected later (during a clinical stable patient phase and recovered). This should be done in an experienced center and signs of exsiccosis should be monitored closely. Generally, diabetes insipidus can be diagnosed if there is no clear increase in urine osmolality (maximum <700 mOsm/kg) or the ratio of peak urine to plasma osmolality is <2. Glucocorticoids may suppress ADH secretion and, thus, diabetes insipidus may be precipitated by glucocorticoid replacement *(Tanriverdi 2015, Arlt 2003, Gasco 2012, Moore 2003, Agha 2004, Rajaratnam 2003)*.

Conclusions

In conclusion, hypopituitarism is a common, potentially serious but treatable complication of TBI, and may also occur for a smaller but significant patient number affected by of SAH and IS. Increased level of awareness among physicians of all disciplines who are involved in the care of patients with TBI, SAH, and SI is required to identify affected cases and provide the appropriate and timely hormone therapy, which has the potential to improve recovery, rehabilitation, and quality of life for those patients.

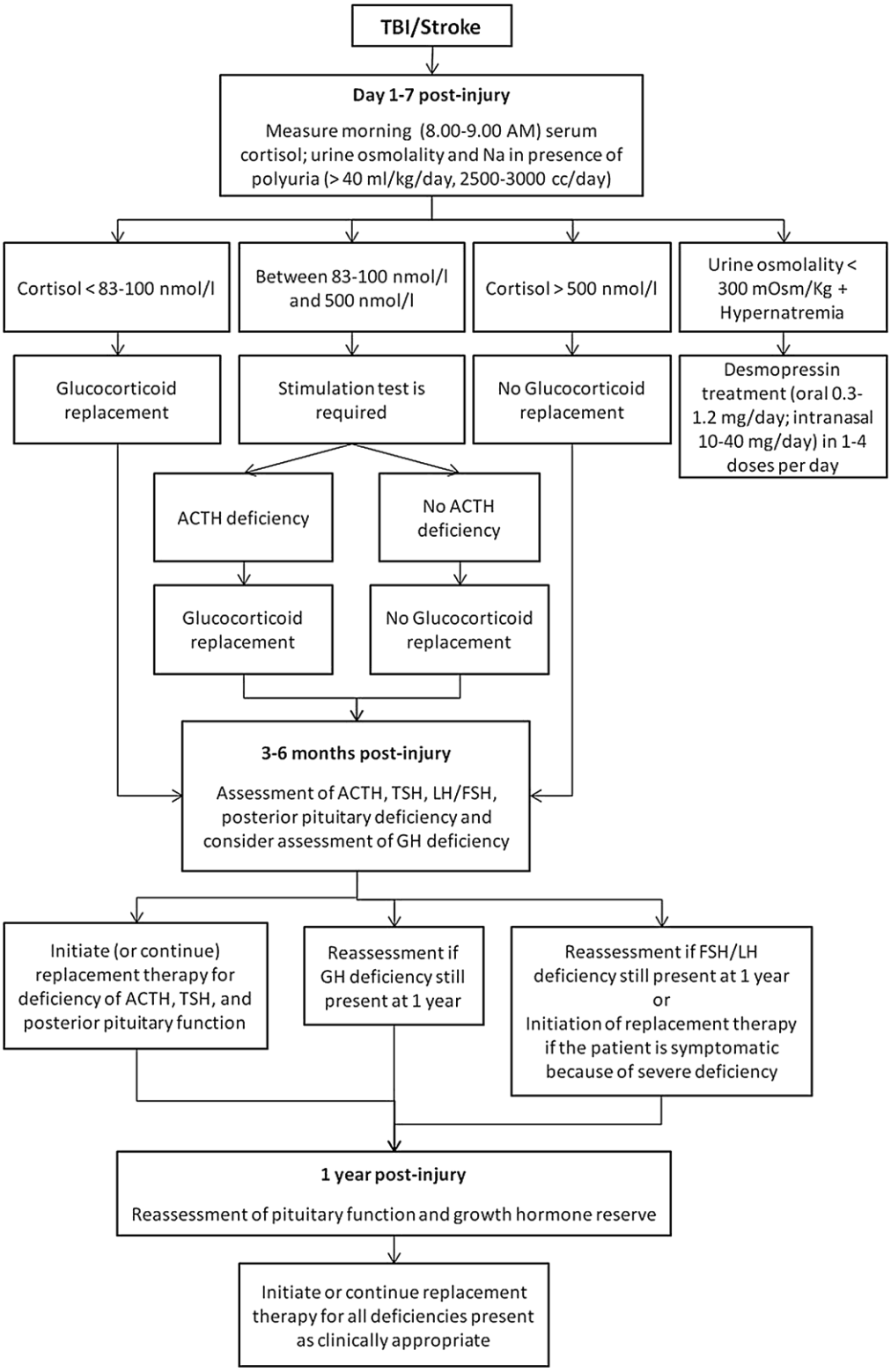
Tables

Table 1. Diagnostic processes of pituitary function in adult TBI/SAH and IS patients.

Endocrine Axis	Diagnostic process	Hormonal Range	Diagnosis
HPA axis	Insulin Tolerance Test (ITT)	Cortisol peak >500 nmol/l (18 µg/dL)	Normal integrity of the HPA axis
	250 or 1 µg ACTH test	Cortisol > 500 nmol/l (>18 µg/dl)	Normal integrity of the HPA axis
		Cortisol ≤ 500 nmol/l (≤18 µg/dl)	ACTH deficiency
	Glucagon test (1 mg im)	Cortisol peak ≥ 500 nmol/l (> 18 µg/dl)	Normal integrity of the HPA axis
	CRH test (100 µg as a bolus ev)	Limited value for the diagnosis of ACTH deficiency	
	MET test	Limited availability of reliable 11-DOC assays	
TRH-TSH-Thyroid axis	TSH and fT4 levels	Low or normal TSH, low fT4	Central hypothyroidism
GnRH-LH/FSH-gonadal axis	PRL (in both sex)	PRL excess should be excluded that may be present due to disturbed hypothalamic inhibition of prolactin release	
	FSH, LH and Estradiol (Premenopause women)	Oligo-amenorrhoea, Estradiol < 100 pmol/l, LH and FSH inappropriately low	Secondary hypogonadism
	FSH and LH (Peri-postmenopause women)	LH and FSH inappropriately low	
	FSH, LH and Testoterone (Men)	Testosterone < 10.4 nmol/l (<3 ng/ml), FSH and LH inappropriately low	
	GnRH test	Is generally not necessary in adults because it does not add greater diagnostic accuracy	
Somatotroph axis	IGF-I	Low age-related IGF-I level	It could be diagnostic "per se" only if all other anterior pituitary hormone are already compromised
	Insulin Tolerance Test (ITT)	Peak GH ≤ 3 µg/l (adults)	GH deficiency
	GHRH+arginine test	BMI related: - BMI < 25 kg/m^2: ≤11.5 µg/l - 25 ≤ BMI > 30 kg/m^2: 8.0 µg/l - BMI ≥ 30 kg/m^2: 4.1 µg/l	
	GHRH+GHRP-6 test	BMI related: - Lean subjects: ≤ 15 µg/l - BMI >35 kg/m^2: ≤ 5 µg/l	
	Glucagon test	GH≤ 3 µg/l	
Posterior Pituitary	Basal urine and plasma sample	Urine volume (≥ 40 ml/kg body weight/ day) + urine osmolality <300 mosm/kg H_20 + hypernatriaemia	Diabetes insipidus
	Water deprivation test	Urine osmolality < 700 mOsm/kg Ratio of urine to plasma osmolality < 2	

Figures

Figure 1. Screening recommendations for pituitary function after traumatic brain injury/stroke.



Use of a new classification algorithm based on administrative health databases to estimate incidence and prevalence of acromegaly in Piedmont Region, Italy.

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Submitted manuscript

Abstract

Background. Recent studies from national registries have described changing patterns in epidemiology of acromegaly. Our study used administrative databases to estimate prevalence and incidence of acromegaly in the Piedmont region, Italy.

Methods. This retrospective study was conducted in Piedmont between 2012-2016 on administrative health databases for inpatients and outpatients of any age. Enrollees were included if ≥2 claims were identified from the following databases: hospital records with acromegaly diagnosis code (ICD-9-CM: 253.0); exemptions from co-payment for acromegaly (code: 001); prescriptions of Octreotide LAR, Lanreotide Autogel, Pegvisomant, Pasireotide LAR; prescriptions of pituitary MRI/CT scans.

Results. 369 individuals (M=146, F=223) met our criteria. Overall incidence was 5.3 per million population per year (95% CI: 4.2-6.7), and prevalence was 83 cases per million inhabitants (95% CI: 75-92). Mean age was 50.9 years. Rates were lowest in ≤19 year olds and highest in middle aged individuals. Both incidence and prevalence were slightly higher among women (Rate Ratio: 1.08, Prevalence Ratio: 1.43). Age-specific incidence was similar between sexes up to 39 years and diverged thereafter, with an increasing trend recorded among men. Prevalence was higher in women aged 40-79 years, and increased continuously up to 79 years in both sexes.

Conclusions. This is the first population-based study conducted in Italy to estimate epidemiology of acromegaly and results show a higher prevalence than previously reported. Although our algorithm requires proper validation, it potentially constitutes a comprehensive tool to describe the pattern of acromegaly.

Background

Acromegaly is a rare entity resulting from a growth hormone (GH)-secreting pituitary adenoma *(Melmed 2009)*. The presentation and initial diagnosis of acromegaly can be insidious and, despite the advances in this field, there are significant diagnostic delays that could prompt adverse sequelae and influence the long-term disease prognosis. Hence, a timely diagnosis is desirable. To increase awareness on the disease, the Endocrine Society guidelines advised to screen for acromegaly in patients with typical clinical manifestations as well as in those without somatic signs but who carry several typical comorbidities of acromegaly, like obstructive sleep apnea, type 2 diabetes mellitus, debilitating arthritis, carpal tunnel syndrome, hyperhidrosis, and arterial hypertension *(Katznelson 2014)*. Similar recommendations were issued in the American Association of Clinical Endocrinologists (AACE) guidelines *(Katznelson 2011)*. Another relevant issue is represented by therapeutic millstones. GH-secreting pituitary tumors display a high prevalence of macroadenomas, which reduces the likelihood of complete surgical cure and prompts persistent disease in over 60% of cases *(Mestron 2004, Starke 2013, Giustina 2014)*. In such instances, multimodal therapies are necessary. Because these diagnostic and prognostic factors impact on the health care systems, it is important that optimal knowledge exists on chronic care activities related to acromegaly, including epidemiology, therapeutics and practices.

Most epidemiological data currently available are extracted from databases collected in specialized referral centers, whose catchment areas may not cover the whole population. Published estimates have suggested highly variable figures encompassing prevalence rates of 28-137 cases per million and annual incidence rates of 2-11 cases per million *(Katznelson 2011, Giustina 2014, Alexander 1980, Bengtsson 1988, Ritchie 1990, Daly 2006, Sesmilo 2013, Burton 2016, Dal 2016, Lavrentaki 2017)*. To improve accurateness in describing the patterns of acromegaly and their impact on healthcare systems *(Lavrentaki 2017)*, recent US studies have employed a novel analytical approach to derive estimates of acromegaly based on data from population healthcare databases and insurance claims *(Burton 2016, Broder 2016, 2017)*. These recent epidemiological data collectively suggest that incidence and prevalence of acromegaly are rising *(Burton 2016, Broder 2016)*. Uncertainty remains on whether this is the result of improved diagnostic tools, stricter

clinical surveillance or temporal changes in incidence rates. The aim of our study was to estimate the prevalence and incidence data on acromegaly from 2012 to 2016 in the Piedmont region through the analysis of routinely collected administrative data.

Methods

Data source

A retrospective cohort study was conducted using the Administrative Health Databases (AHDs) of Piedmont Region (about 4.400.000 inhabitants, corresponding to 7.5% of the national population), from January 1st, 2012 to December 31st, 2016. Data were obtained from four different databases consisting of: a) Drug Claims Registry, including records of all outpatient drug prescriptions reimbursable by the National Health Service (NHS); b) Hospital Information System, including records of all hospital discharge forms (HDFs) from public or private hospitals, containing all clinical data related to hospital discharge and day surgeries; c) Co-payment Exemption Registry; d) Outpatient Specialist Service Information System, reporting records of endocrine visits and healthcare services related to the workup of acromegaly provided by the NHS (laboratory and medical tests, outpatient visits, neuro-imaging). These four databases can be linked through a unique identifier, which remains unchanged over time. The medical claims in HDFs included information on diagnoses reported according to the International Classification of Diseases, 9th Revision, Clinical Modification (ICD-9-CM) diagnosis codes. Laboratory and radiological data included information reported according to the National Tariff Nomenclature (NTN) codes.

Selection of cases

Potential cases were defined as the subjects who had claims suggestive of acromegaly in at least two of the four databases during the study period. The claims taken into account were:

1) Hospital discharge records with an acromegaly diagnosis code (ICD-9-CM: 253.0);
2) Exemptions from co-payment for acromegaly (code: 001);

3) Prescriptions for any of the following medications: octreotide (ATC: H01CB02), lanreotide (ATC: H01CB03), pegvisomant (ATC: H01AX01), pasireotide (ATC: H01CB05);
4) Prescriptions for any of the following radiological tests: facial bones nuclear magnetic resonance (NMR) (codes 88.91.3-88.91.4); cranial (sella turcica, orbit) computed tomography (CT) (codes 87.03-87.03.1).

To further improve the specificity of our approach, we did not take into considerations drug prescriptions if:

1) Patients had received less than three separate drug prescriptions for the treatment of acromegaly (occasional drug users);
2) The medications were not long-acting release (LAR) formulations (octreotide LAR, lanreotide Autogel, pasireotide LAR);
3) Patients taking octreotide or lanreotide had a hospitalization with a diagnosis different from acromegaly, among those for which there is an indication for the use these drugs, as reported in the summary of product characteristics (malignant neoplasms (ICD-9: 140-209; 230-239), liver disorders (ICD-9: 570-573), gastrointestinal bleeding (ICD-9: 578), esophageal varices (ICD-9: 42), Cushing's disease (ICD-9: 255; 255.0). 4) Patients had an exemption for Cushing's disease (code: 032).

Statistical analysis

Patients were stratified by age (≤19, 20-39, 40-59, 60-79, and ≥80 years) and sex. Incidence was calculated by dividing the number of new acromegaly cases (i.e., no evidence of acromegaly during the two years prior to the index claim) by the size of the population total time at risk during each calendar year. Prevalence calculated by dividing the number with an acromegaly-related claim, during each calendar year or any time prior, by the total number of residents in Piedmont for the entire calendar year. Incidence and prevalence estimates were stratified by age decades and sex. Comparisons of incidence and prevalence between the two sexes were carried out estimating rate ratios and prevalence ratios. Analyses were performed with the software Stata 12 (StataCorp, College Station, TX, USA).

Results

In the period 2012-2016, we found 369 individuals in the Piedmont population, 146 males and 236 females, who met our combined criteria for diagnosis of acromegaly. Incident cases in the period 2014-2016 were 71, of whom 33 were men and 38 were women. Mean age was 50.9 years. The observed number of cases translated into an incidence of 5.3 per million per person year (py) (95% CI: 4.2-6.7) and a prevalence of 83 per million inhabitants (95% CI: 75-92) (Table 1, pag. 263). When the index cases were stratified by sex, both the incidence and the prevalence were slightly higher among women. Compared to men, the Rate Ratio of women was 1.08 (95% CI: 0.67-1.72), while the Prevalence Ratio was 1.43 (95% CI: 1.16-1.76). Rates were lowest among subjects with less than 19 years old. Age-specific incidence rates were similar in the two sexes up to the age of 39 years but seemed to diverge thereafter, with an increasing trend recorded among men as compared to women (Figure 1, pag. 264). Prevalence increased in both sexes continuously up to 79 years of age, and markedly decreased thereafter. Prevalence of acromegaly was substantially higher in women than men between 40 and 80 years of age (Figure 2, pag. 265).

Discussion

This is the first population-based study conducted in Italy to estimate the prevalence and incidence of acromegaly in a geographically defined population without apparent limitations related to catchment area and referral practice. In order to do so, we developed an algorithm that identifies acromegaly cases combining information from four different administrative databases. This approach is similar to that adopted by Burton et al. *(Burton 2016)* and Broder et al. *(Broder 2016)*, who used two large health insurance databases to estimate the incidence of acromegaly in the US. In addition to medical and pharmacy claims, however, our algorithm takes also advantage from information included the registry of co-payment exemption and hospital discharge records, two reliable sources of data that have been previously used for epidemiological research in Italy. Our study showed an overall incidence of acromegaly of 5.3 cases per million py, while the

prevalence was 83 cases per million inhabitants. While the incidence of acromegaly here reported is comprised within the highly variable range of 1.9-7.7 cases per million py found in European studies *(Bengtsson 1988, Dal 2016, Sesmilo 2010, Bex 2007, Reincke 2006, Tjörnstrand 2014, Arnardottir 2014, Hoskuldsdottir 2015, Agustsson 2015)*, two recent US surveys have reported a higher incidence rate corresponding to 9.6-11.7 cases per million py *(Burton 2016, Broder 2016)*. The discrepancy between these and our estimates suggests potential differences in study methodologies, and the implicit possibility that acromegaly incidence may differ across the world. Mean age at the diagnosis of acromegaly was 50.9 years, we observed a trend toward an increased incidence with increasing age, which is aligned with recent reports *(Lavrentaki 2017)*. Although the overall incidence was similar between sexes, an age-specific analysis showed trends of incidence were similar between sexes up to the age of 39 years but slightly increased in men thereafter, as compared to women. These results are consistent with those obtained in the Swedish Pituitary Registry, which displayed an increasing trend for men and a decreasing one for women after the age of 35 years *(Tjörnstrand 2014)*.

With regard to prevalence rates, we observed an age-dependent increase in both sexes, leading to a prevalence peak in the age group 60-79 years. The prevalence of 83 cases per million inhabitants found herein is higher than that of 60 cases per million recorded in a large Italian survey *(Arosio 2012)*, as well as that obtained in other retrospective European studies *(Katznelson 2011, Bex 2007)*. However, our estimates parallel the prevalence rates documented in more recent surveys *(Burton 2016, Dal 2016, Broder 2016)*, suggesting that the use of combined database could improve the description of prevalence figures. Of note, a recent Icelandic survey suggested that prevalence could actually be underestimated, reaching in such study a rate of 121 cases per million py *(Hoskuldsdottir 2015)*. Our study also showed a higher prevalence of acromegaly in women compared to men up to the age of 80 years, whereas the opposite was documented thereafter.

It is still a matter of debate whether incidence rates of acromegaly are increasing over time, and whether this is a real phenomenon or it is rather attributable to improved diagnostic techniques and disease awareness. Likely, the development of sensitive immunoassays for measuring GH and IGF-1 levels, as well as the widespread use of MRI for detecting small pituitary tumors, has increased the number of diagnoses of

acromegaly over time *(Ribeiro-Oliveira 2012)*. In fact, many of the earlier studies were performed when neuroimaging technology could only allow the identification of large tumors, while highest resolution and vast use of MRI now enables identification of pituitary masses that, previously, would have been too small to detect *(Heitkamp 2014)*. For this reason, incidentally discovered pituitary mass can be now identified in patients undergoing neuroimaging for unexplained headache, head injury workup, sinus disease, cervical spine disease, and vertigo. Once the diagnosis of pituitary mass is made, clinical evaluation often include complete assessment of pituitary function to uncover an otherwise clinically silent disease *(Freda 2011)*. Also, current diagnostic criteria could have contributed to the increase of incidental diagnosis of acromegaly, as they no longer require anymore the presence of typical phenotypic features of acromegaly *(Katznelson 2014)* or visible pituitary tumors on MRI *(Lonser 2010)*. Finally, increased awareness of the condition and its early manifestations may also have contributed to the apparent increase in disease prevalence.

Population-based design, definite regional enrollment, and multiple database sources are major strengths of our study. Results from this type of epidemiologic study are more generalizable than estimates derived from single-institution studies or case series *(Lavrentaki 2017)*, and could compensate for the lack of national registries. Moreover, our study included all age groups, as compared to other capturing commercially-insured patients under the age of 65 years *(Broder 2016)*. A limitation of using claims data to estimate disease incidence is the inability to know with certainty that the first diagnosis seen in the data represents the first clinical diagnosis of the condition. Moreover, this approach cannot provide information on time from diagnosis and disease status (e.g., active vs. inactive disease), as well as it lacks information on adenoma size, hormone levels, clinical stratification of subgroups, and cases of multiple endocrine neoplasia-1. Finally, but more importantly, our algorithm requires validation to constitute an effective tool.

Conclusions

In conclusion, this population-based study provides a novel method to estimate the incidence and prevalence rates of acromegaly at the population level. Current results are

consistent with the available literature on this topic and show a higher prevalence of acromegaly than previously reported. Even if our algorithm requires proper validation and larger assessments to detail the epidemiology of acromegaly in Italy, it could represent a comprehensive tool to describe the pattern of acromegaly, to assess its burden on patients and health care systems, and to provide guidance on resources allocation, especially in countries lacking national registries on acromegaly. Future developments include the assessment of time trends of the disease and the extension of the study to other Italian regions to evaluate geographical homogeneity.

Tables

Table 1. Prevalence (period 2012-2016) and incidence (period 2014-2016) of acromegaly in Piedmont region (Italy), stratified by sex. Results expressed as cases per million inhabitants.

	Men		**Women**	
	N	Point estimate (95% CI)	N	Point estimate (95% CI)
Incidence	33	5.1 (3.6-7.2)	38	5.5 (4.0-7.6)
Prevalence	146	68 (58-80)	223	97 (85-111)

Figures

Figure 1. Incidence of acromegaly in Piedmont region (Italy) in the period 2014-2016, stratified by sex and age groups.

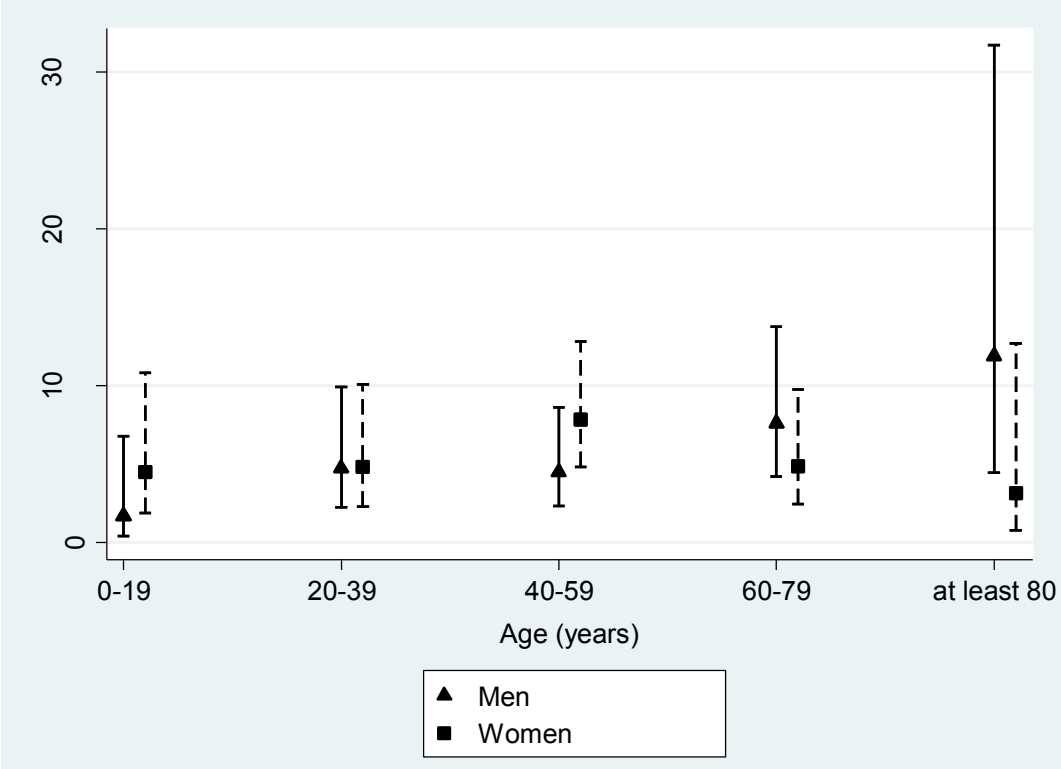
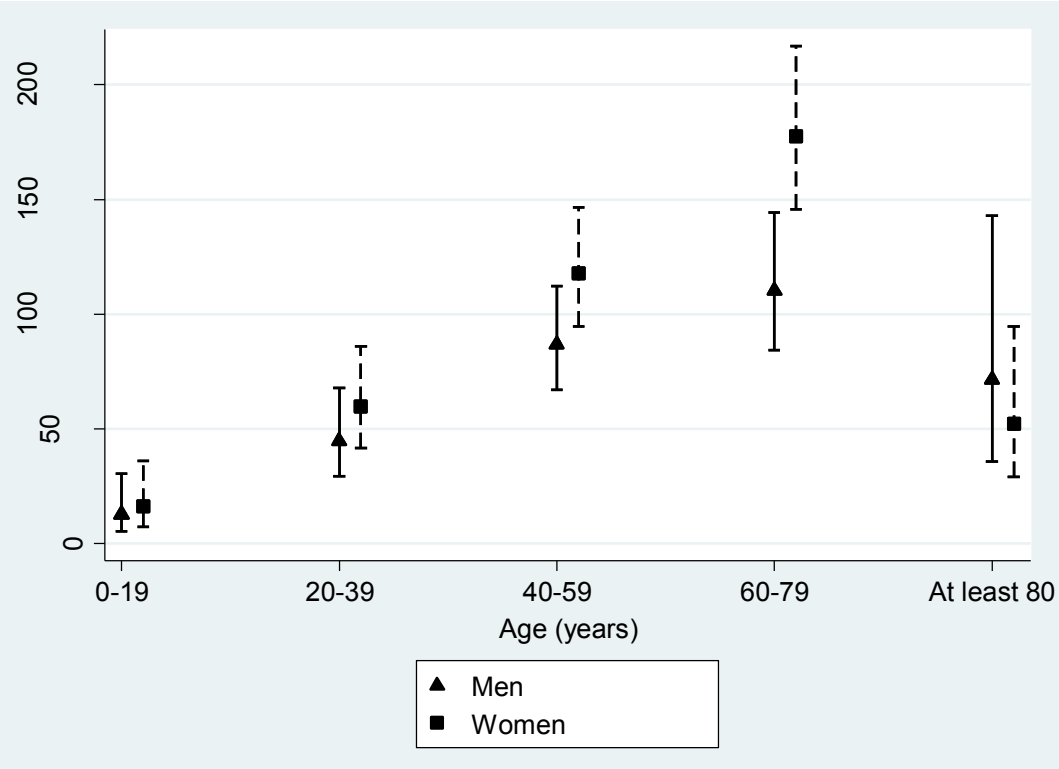


Figure 2. Prevalence of acromegaly in Piedmont region (Italy) in the period 2012-2016, stratified by sex and age groups.



VITAMIN D AND NEUROLOGICAL DISEASES

Introduction

Vitamin D is a secosteroid hormone typically associated with regulation of phosphocalcic homeostasis and osteogenesis *(Kalueff 2007)*. The past decade was characterized by an increased number of publications which highlighted that vitamin D was not only associated with bone disease, but also with non-bone adverse health outcomes, including immunosuppression, cancer, infections, and cardiovascular diseases *(Kalueff 2007, Zitterman 2003)*. These associations are related to the fact that vitamin D has multiple biological targets mediated by the vitamin D receptor (VDR) present in numerous cells *(Kalueff 2007)*.

Vitamin-D-related effects upon the central nervous system (CNS) have been described less than other target organs *(Kalueff 2007)*, but it appears that vitamin D deficiency is associated with neurological dysfunction *(Kalueff 2007, Zitterman 2003)*.

This section aims to provide an endocrine overview on the relationship between vitamin D and neurological diseases.

Vitamin D and Neurological Diseases: An Endocrine View

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Abstract

Vitamin D system comprises hormone precursors, active metabolites, carriers, enzymes, and receptors involved in genomic and non-genomic effects. In addition to classical bone-related effects, this system has also been shown to activate multiple molecular mediators and elicit many physiological functions. In vitro and in vivo studies have, in fact, increasingly focused on the “non-calcemic” actions of vitamin D, which are associated with the maintenance of glucose homeostasis, cardiovascular morbidity, autoimmunity, inflammation, and cancer. In parallel, growing evidence has recognized that a multimodal association links vitamin D system to brain development, functions and diseases. With vitamin D deficiency reaching epidemic proportions worldwide, there is now concern that optimal levels of vitamin D in the bloodstream are also necessary to preserve the neurological development and protect the adult brain. The aim of this review is to highlight the relationship between vitamin D and neurological diseases.

Background

The vitamin D system comprises steroid pro-hormones, their metabolites, carriers and enzymes involved in vitamin D metabolism *(Norman 1998)*. Vitamin D occurs in nature as two main forms: vitamin D2 (ergocalciferol), which is photochemically synthesized in plants, and vitamin D3 (cholecalciferol), which is synthesized in the skin after exposure to sunlight, in particular ultraviolet B radiation at an appropriate wavelength of 280–320 nm. Vitamin D metabolism is complex and tightly regulated *(Norman 1998)*. The classical synthetic vitamin D pathway involves sequential 25-hydroxylation [25(OH)D] and 1α-hydroxylation [1,25(OH)2D] of vitamin D2 and D3 precursors in the liver and kidney, respectively. Vitamin D metabolites circulate in the bloodstream as bound to the vitamin D binding protein (DBP). Vitamin D catabolism is mostly mediated by 24-hydroxylase (CYP24A1), which converts 1,25(OH)2D (calcitriol) in 1,24,25(OH)3D. This catabolite binds with significantly lower affinity the vitamin D receptor (VDR), and is further processed into excretion products (calcitroic acid). Classically, vitamin D has a recognized role in the regulation of bone health physiology and calcium-phosphorus homeostasis, by acting at the level of the skeletal bone, intestine and kidney. There is now consistent evidence showing that several "non-calcemic" effects of vitamin D metabolites occur in vitro and in vivo, and increasing consideration is given to vitamin D status as a marker of general health, since low vitamin D levels are associated with the development and progress of autoimmunity, infectious diseases, diabetes mellitus, cardio-metabolic disorders, obesity, neuromuscular disorders, and cancer *(Holick 2008)*. Vitamin D and its congeners yield both genomic and non-genomic actions. Genomic actions are mediated by the VDR, a member of the steroid hormone superfamily. The VDR is a nuclear receptor present in over 30 human tissues, which regulates approximately 3% of the human genome (i.e., about 700 genes) *(Pertea 2010, Ramagopalan 2010, Pannu 2016)*. Nuclear VDRs are present in the majority of cells of the body and form the basis for the investigations in the extra-skeletal benefits of vitamin D.

VDR, as well as 25-hydroxylase and 1α-hydroxylase, the enzymes controlling vitamin D activation, along with 24-CYP24A1, the enzyme controlling vitamin D degradation, are expressed in the brain *(Garcion 2002, Balden 2012, Eyles 2005)*. The main consequence is that the central nervous system (CNS) is able to synthesize its own vitamin D, which yields

auto- or paracrine neurosteroid actions at the local level *(Eyles 2005)*. Neurons and glial cells, particularly in the temporal, cingulate, and orbital cortices and in the thalamus, nucleus accumbens, stria terminalis, and amygdala, express the VDR and 1α-hydroxylase *(Kalueff 2007)*. The distribution of the VDR and 1α-hydroxylase has also been investigated in the adult human brain, and is similar to that found in the rat *(Eyles 2005)*. Calcitriol-ligand binding to VDR allows heterodimerization with retinoid X receptor (RXR) and its ligand (9-cis-retinoic acid), then the VDR/RXR complex binds specific sequences in the promoter region of vitamin D responsive genes (vitamin D response elements; VDREs). Vitamin D actions on brain development involve effects on cellular proliferation, differentiation, calcium signalling, neurotrophism and neuroprotection. It also appears that vitamin D plays a role in neurotransmission and synaptic plasticity, and a link has been described between vitamin D and dopaminergic neurotransmission *(Cui 2010, Cui 2013, Kesby 2010)*. Collectively, the physiological effects of vitamin D in brain functions include the promotion of neurotransmission, neurogenesis, synaptogenesis, amyloid clearance and the prevention of neuronal death. It is, thus, not surprising that observational studies have documented associations between higher serum vitamin D concentrations and healthier cognitive performance *(Annweiler 2016)*.

Another recent field of interest regards VDR gene polymorphisms. Preliminary data suggest that single nucleotide polymorphisms (SNPs) in the VDR gene may have roles in the development of multiple sclerosis, Parkinson's disease, and Alzheimer's disease *(Tizaoui 2015, Lee 2014)*. However, recent results on the association between VDR gene polymorphisms and different neurological diseases are somewhat contradictory, and the role of VDR in the aetiology of neurological diseases is still uncertain. Further investigations are needed to obtain more definitive results.

More than 85% of circulating 25(OH)D and 1,25(OH)2D, the bioactive metabolite of vitamin D, are tightly bound to the DBP, which circulates in molar excess with respects to its ligands. As little as 5% of all circulating DBP is bound to vitamin D metabolites. DBP can also bind globular actin (G-actin) with high affinity and sequesters G-actin released into the circulation upon tissue/cell damage or necrosis, thus participating to the organism's actin scavenging system *(Meier 2006)*. This peculiar action confers DBP a vital role, since circulating free G-actin polymerization into long filaments can initiate disseminated intravascular coagulation (DIC) if not rapidly cleared. DBP also intervenes to regulate the

chemotactic activity of complement 5a (C5a), and it partakes in the inflammatory cascade as a precursor of macrophage activation factor (MAF), which originates from DBP through modifications of the glycosylated residues *(Nagasawa 2005)*. The "free hormone hypothesis" infers that DBP works as a reservoir and delivery system for free vitamin D metabolites to target tissues at the cellular level *(Nagasawa 2005)*. The free hormone hypothesis states that the biological activity of a given hormone is affected by its unbound, rather than protein-bound, concentration in the plasma. It has been suggested that the free hormone hypothesis could exist occur even if tissue uptake is caused by a mechanism involving one or more circulating protein-bound pools of hormone, as well as for the hydroxylated metabolites of vitamin D. Nevertheless, endocytic receptors have also been identified which are capable of transporting DBP-vitamin D complexes inside the target cells *(Mendel 1989)*, and are reckoned as essential for the renal metabolism of vitamin D. Of note, DBP-dependent transport mechanisms are also thought to contribute to vitamin D access to the CNS *(Smolder 2011)*. Given the multifaceted activities of DBP, an alteration of its circulating levels may impact pathophysiology in different ways, one of which could imply modifications of vitamin D bioavailability.

Non-genomic actions of vitamin D have been discovered in many systems, and only recently they have been identified in the brain *(Cui 2017)*. Non-genomic pathways cooperate with the classical genomic pathway to trans-activate the VDR and exert the effects of calcitriol. Non-genomic signalling is rapid, does not depend on transcription and may indirectly affect transcription via cross-talk with other signalling pathway. Data suggest that non-genomic actions of vitamin D occur at the plasma membrane level and involve a non-classical membrane-associated receptor, and a calcitriol-membrane-associated rapid response steroid binding protein (1,25-MARRS) *(Norman 2006, Nemere 2004)*. Also, non-genomic actions of calcitriol induce calcium translocation across intestinal membranes, and calcitriol binding to membrane receptor activates signalling cascades leading to an increase in intracellular calcium flux via opening of voltage-gated calcium channels. This may, in turn, activate other growth regulatory pathways (e.g., rat sarcoma family of GTPases (RAS), murine leukemia viral oncogene (Raf)-1, mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPK/ERK), as described in skeletal muscle cells). Moreover, ERK can enhance transcriptional activity of the VDR and non-genomic activation of protein kinase C (PKC) may stabilize VDR via

phosphorylation *(Morelli 2001, Wali 1990, Hsieh 1991)*. There is also evidence that 1,25(OH)2D modulates l-type calcium channel (LTCC) functions, and these effects can influence neuronal function *(Cui 2015)*, such as neuronal maturation during developmental stages and/or neuroprotection during adulthood *(Zanatta 2012, Bigos 2010)*.

As summarized in Figure 1 (pag. 291), vitamin D deficiency has been mechanistically and clinically linked to neurological diseases and neuropsychological disorders, cognitive impairment and neurodegenerative diseases *(Cui 2017, Groves 2014, FNB 1997, Luque-Cordoba 2017, McGrath 2010, Vinkhuyzen 2016, Landel 2016)*. While vitamin D deficiency may act as a common risk factor *(Groves 2014)*, it should be borne in mind that the origin of these disorders is often complex and involves both genetic and environmental causes. Interestingly, animal and human data have suggested that vitamin D status, particularly vitamin D deficiency, may intervene during adulthood to modulate the exacerbation of inherent brain disorders and/or impair the recovery from brain stressors *(Groves 2014)*. It should be mentioned here that vitamin D deficiency has also been related to mood disorders and a number of psychiatric conditions. While some cross-sectional and epidemiologic studies have found that low levels of vitamin D are significantly associated with higher levels of depressive symptoms or with a depression diagnosis, data are currently argued for the lack of causality, that is, current evidence does not definitively demonstrate that vitamin D deficiency is a cause of or risk for developing depression nor that vitamin D is an effective therapy for depression. For these reasons, we reckon that this topic should be distinctly addressed in further reviews.

One key aspect related to vitamin D homeostasis and neurological disorders is the role of vitamin D supplementation in 25(OH)D deficient patients. Results of clinical trials conducted in patients with neurological disorders have, so far, provided controversial findings *(Cui 2017, Landel 2016, Avenell 2016, Dong 2016, Rautiainen 2016, Sanders 2016, Winzenberg 2016)*, and the potential confounding effect of baseline vitamin D status and supplementation doses exists. It is of note that interventional studies conducted in the general population treated with vitamin D have mostly focused on the relationship between achieved vitamin D levels and the prevention of falls and fractures *(Dawson-Hughes 1997, Chapuy 2002, Trivedi 2003, Sanders 2010)*. At present, the Institute of Medicine *(Ross 2011)* recommends attaining 25(OH)D concentrations ranging between 20

and 50 ng/mL during vitamin D supplementation, whereas the Endocrine Society, the International Osteoporosis Foundation, and the American Geriatric Society suggest that a minimum level of 30 ng/mL is needed to minimize the risk of falls and fracture in older adults *(Holick 2011, Dawson-Hughes 2010, AGS 2014)*. For concentration purposes: to convert ng/mL to nmol/L: 1 ng/mL = 2.5 nmol/L; for dosing purposes: to convert mcg to IU: mcg/0.025 = IU. The Institute of Medicine systematic review also voiced concern for serum 25(OH)D concentrations above 50 ng/mL *(Ross 2011)*. These concerns were based on an increased risk of fractures and certain types of cancer (pancreatic and prostate) in people receiving high dose vitamin D supplementation *(Sanders 2010)*. In general, vitamin D should be supplemented to reach 25(OH)D level of at least 20 ng/mL, and a level of 30 ng/mL is recommended by most guidelines *(Holick 2011, Dawson-Hughes 2010, AGS 2014)*. A critical 25(OH)D threshold of 200 ng/mL should be mandatorily avoided to reduce the risk of hypercalcemia and symptoms of vitamin D excess. Expectedly, the required dose of vitamin D supplements varies between individuals depending on baseline 25(OH)D level, seasonality, latitude, ethnicity, nutrition, adiposity, dosing and type of vitamin D analog used for supplementation *(Mazahery 2015)*. The optimal approach for supplementation in the general population, and in patients with neurological diseases, has not been established yet.

For many reasons, therefore, vitamin D supplementation in neurological diseases needs to be optimized in terms of dosing and timing to generate a therapeutic potential. Recent studies suggest that vitamin D supplementation doses should be individualized depending on baseline vitamin D status and responsiveness. The potentially interfering activity of factors such as adiposity, enzymes catalyzing the different pathways *(Cui 2017, Groves 2014, Luque-Cordoba 2017)* and VDR genotypes should be appropriately addressed *(Carlberg 2016)*. Large, randomized, well-controlled clinical trials are also awaited to provide evidence on the safety and tolerability of vitamin D as adjunct therapy. The aim of this review is to provide an unbiased overview on the relationship between vitamin D and neurological diseases.

Multiple Sclerosis and Vitamin D

Multiple sclerosis (MS) is a debilitating slow progressive disorder of the central nervous system, which is characterized by axonal injury and demyelination in the spinal cord and brain. Although its etiology is unclear, it seems to be multifactorial, with dysregulation of the immune response, environmental factors and genetic determinants all playing a contributive role *(Van Amerongen 2004)*. In fact, genetic, epidemiological and immunological studies showed that MS is an autoimmune disease *(Ebers 1996, Oksenberg 2001)* influenced by environmental risk factors such as infections, cigarette smoking, obesity, and inadequate serum levels of vitamin D and/or its metabolites *(O'Gorman 2012, Pugliatti 2008, Ascherio 2007)*. Attention has been recently given to the study of the microbiome in MS and its relationship with environmental stressors *(Van Amerongen 2004, Tremlett 2017)*. Emerging cross-sectional, case-control studies have shown that microbiota composition differs between MS subjects and controls *(Jangi 2016, Chen 2016)*. Nevertheless, human studies supporting the role of microbiota in MS are still scarce.

Of the environmental factors identified in association to MS risk and capable of influencing its clinical course, vitamin D is among the strongest and most consistently found in connection *(Ascherio 2010, Simpson 2010, Ruina 2012, Munger 2006, Mowry 2010)*. A direct relationship has been observed between latitude and the prevalence of MS, which suggests a role for UV radiation and vitamin D in MS development *(Simpson 2011)*. Other studies showed that the risk of MS decreases with increasing intake of vitamin D *(Munger 2004)*, and serum 25(OH)D levels are significantly lower in patients with MS as compared to healthy controls *(Mazdeh 2013)*. Genetic studies found a relationship between MS susceptibility and SNPs of enzymes genes relating to vitamin D metabolism, namely, CYP27B1 and CYP24A1 *(Ramagopalan 2011, IMSGC 2011)*. Genome-wide association studies have identified more than 100 non-human leucocyte antigen (HLA) genetic risk loci, many acting as cooperative networks. However, each of these individual loci exerts modest influence on MS risk, and major histocompatibility complex (MHC) remains the key susceptibility locus *(Gourraud 2012)*. Other studies also focused on VDR gene polymorphisms in association with increased susceptibility to develop MS, or with modulation and progression of MS. As such, significant associations were

obtained for the VDR gene polymorphisms Bsm-I in a Japanese cohort, Apa-I in Japanese and Australian cohorts, and Taq-I in an Australian cohort *(Fukazawa 1999, Niino 2000)*. Further, a trend for the polymorphism Fok-I was described in a UK cohort study *(Partridge 2004)*, while a Canadian study found no preferential transmission of Apa-I and Taq-1 from MS-affected parents to affected offspring, and no association between Taq-I polymorphism and MS was found in another UK study *(Partridge 2004, Steckley 2000)*. Genetic data also suggest that vitamin D may act on the risk of MS by influencing the regulation of vitamin d-responsive genes involved in immunity, such as the HLA-DRB1* 1501 allele, which has a highly conserved VDR responsive element in its promoter *(Gourraud 2012, Agliardi 2011)*.

Vitamin D influences the cytokine profile and the inflammasome *(Smolders 2008)*. In vitro studies support the evidence that vitamin D prevents interleukin (IL) 2, IL12 and interferon-gamma production, as well as B cells production *(Smolders 2008, Chen 2007, Meehan 2002)*. In addition, vitamin D negatively regulates TH17-mediated autoimmune diseases, one example of which is MS *(Chang 2010)*. Activation of the VDR by vitamin D stimulates a shift from proinflammatory Th1 responses to anti-inflammatory Th2 responses in brain *(Smolders 2008)*. Proliferation suppression assays showed an association between high 25(OH)D levels and improved regulatory T cell function in patients with MS *(Correale 2009, Smolders 2009)*. Interestingly, while the Epstein-Barr virus (EBV) infection appears to be a necessary (but insufficient) condition for adult MS to develop *(Goodin 2009)*, low vitamin D could act on the immune response to EBV to increase the risk of MS *(Holmoy 2008, Hayes 2008, Grant 2008)*. Therefore, a role for vitamin D in the immune regulation of MS is, for many reasons, biologically plausible.

From a clinical viewpoint, several observations suggest a role for vitamin D in MS. Evidence relating MS to impaired vitamin D status is prompted by studies conducted both in children and adults from Australia *(Van der Mei 2007)*, United States *(Munger 2006, Mowry 2010, Mahon 2003, Nieves 1004, Weinstock-Guttman 2004)* and Europe *(Soilu-Hanninen 2008)*. Common associations involve circulating 25(OH)D levels, geographic distribution of MS, bone mass density in MS patients, seasonal fluctuations of 25(OH)D, parameters of MS disease, MS births, MS course in pregnancy, and results of genetic analysis on the VDR. As such, a matched case-control study using neonatal dried blood spots samples from 521 patients with MS outlined an association between 25(OH)D levels

and MS risk as evaluated by odds ratios (ORs), showing a higher MS risk in individuals in the bottom 25(OH)D quintile compared to the top quintile (OR, 0.53), whereas a 25 nmol/L increase in neonatal 25(OH)D resulted in a 30% reduced risk of MS (OR, 0.70) *(Nielsen 2017)*. In a Finnish study on vitamin D levels in over 1000 pregnant women who were later diagnosed with MS and over 2000 comparable women without MS, a two-fold increased MS risk was found among vitamin D–deficient women compared with vitamin d-replete women, and each 50 nmol/L increase in vitamin D level was associated with a 39% lower risk for MS *(Munger 2017)*. Inversely, other studies failed to demonstrate direct associations between MS and vitamin D, whereas one study found low 25(OH)D in male but not female patients with MS *(Orton 2008, Kimball 2007, Tuzun 2003)*.

Sun exposure is, indeed, the most important predictor of vitamin D status. In retrospective studies, sun exposure during childhood and adolescence was associated with a lower risk of adult-onset MS *(Van der Mei 2003, Kampman 2007, Islam 2007, Dalmay 2010)*, although these surveys are potentially challenged by the accuracy of recall. Studies in individuals migrating from the tropics to temperate regions before or during adolescence also found an increased risk of MS *(Cabre 2007)*. Thus, it could be hypothesized that low sun exposure at young ages could impair vitamin D status, hence increase the risk of MS later in life. However, scant data on the link between 25(OH)D and MS exist in world regions where MS is extremely rare, such as peri-equatorial countries, Africa, and a few Asian regions. Evidence of vitamin D insufficiency in the rare cases of MS diagnosed in these regions would give strength to the hypothetical association between vitamin D status and MS.

Brain imaging studies have shown that vitamin D influences the disease activity in patients with MS and relapse risk *(Simpson 2010)*. In relapsing-remitting MS (RRMS), the severity of vitamin D insufficiency was related to higher levels of disability measured by the Expanded Disability Status Scale (EDSS), and was greater in patients with progressive forms of MS as compared with RRMS, suggesting that vitamin D status could have a prognostic value in MS *(Van der Mei 2007, Smolders 2008)*. However, this link was not confirmed in other studies including fewer patients *(Harandi 2012, Hatamian 2013)*.

It is also interesting to reckon that some disproportion has been noticed in the female/male ratio of MS patients with relapse-onset disease (relapsing remitting (RR) and secondary progressive (SP)). An association between skin type and MS-related disability

has only been documented in female patients *(Woolmore 2007)*, implying a gender-related effect in vitamin D metabolism, while a rodent study found an association between dietary vitamin D and inhibition of severe experimental autoimmune encephalomyelitis (EAE) in female but not male mice *(Spach 2005)*, suggesting a gender-based difference in vitamin D responsiveness. Oppositely, a recent prospective study on 101 patients and 107 controls followed during one year with 25(OH)D and 1,25(OH)2D measurements in summer and winter *(Kragt 2009)* documented no difference in vitamin D levels between groups, yet an association between high levels of 25(OH)D and lower incidence of MS and MS-related disability could only be observed in the female population. However, the processes relating to clinical manifestations of MS, such as inflammation, demyelination, axonal damage and repair mechanisms, is unequally distributed across patients and genders, therefore suggesting the intervention of interacting factors relating to the disease and response to treatments *(Comabella 2014)*.

Despite the extensive literature focusing on cerebro-spinal fluid (CSF) biomarkers in MS, only qualitative and quantitative biochemical methods have been used to assess intrathecal production of immunoglobulins *(Axelsson 2013)*. Considering the multiple roles of DBP, which include actin sequestration and a range of less-defined roles in modulating immune and inflammatory responses (*Meier 2006, Chun 2012)*, several studies have suggested a potential modulatory role for DBP in the development of MS *(Qin 2008, Lehmensiek 2007, Stoop 2010, Ottervald 2010, Kroksveen 2012, Disanto 2010)*, although DBP results in CSF or plasma have generated discrepant results *(Qin 2008, Ottervald 2010, Smolders 2013, Kulakowska 2008, Kulakowska 2010, Yang 2013, Rinaldi 2015)*. Recently, Perga et al. proposed a novel biomolecular tool consisting of two isoforms of DBP and ApoE in CSF, which may aid monitoring the progression of MS *(Perga 2015)*. If validated in a larger population, this tool could provide insights on clinical management, treatment strategy and long-term prognosis of MS patients. Likewise, studies on DBP glycosylation and the activity of the enzymes involved in glycosylation/deglycosylation could assist researchers in deciphering the pathogenetic pathways associated with the onset, progression and response to treatments in MS patients.

There are studies suggesting that vitamin D supplementation could be used therapeutically for subjects with MS or those at a risk for MS. The optimal approach for

vitamin D supplementation in the general population, and in patients with MS, has not been convincingly established, with controversies relating to dose, time and target levels of vitamin D treatment *(Ross 2011, Holick 2011, Dawson-Hughes 2010, AGS 2014)*. The relationship between target vitamin D levels and modulation of the risk/progression of MS may differ from that used for skeletal health *(Rosen 2011)*, and vitamin D supplementation trials in MS have shown inconclusive outcome results *(Luque-Cordoba 2017)* due to the lack of long-term follow-up and methodological bias. Preliminary studies inferred that high-dose vitamin D supplementation is generally well tolerated in MS patients. Phase I and phase II trials using high-dose vitamin D3 supplementation have shown that patients with MS can tolerate doses as high as 20,000 IU daily for 12 weeks *(Smolders 2010)*, as well as escalating doses of vitamin D3 up to 40,000 IU daily over 28 weeks *(Burton 2010)*, with barely detectable side effects. However, findings obtained in short-term studies await more definitive evidence to prove the safety of long-term, high-dose vitamin D supplementation. Longer phase II or phase III randomized clinical trials (RCTs) are ongoing and will provide more evidence regarding the safety and tolerability of vitamin D as an adjunct therapy *(Smolders 2011, Dorr 2012, Bhargava 2014)*. In a recent review, Pierrot-Deseilligny et al., *(Pierrot-Deseilligny 2017)* suggested a pragmatic and practical approach for vitamin D3 supplementation using moderate oral doses (between 2000 and 4000 IU/day) for all types of MS patients, including pregnant women *(Wagner 2017)*. These authors underlined the advantages of supplementation doses: (1) the correction of vitamin D insufficiency existing in the great majority of MS patients, with 25(OH)D serum levels thus increasing up to the currently recommended range (30–60 ng/mL) *(Pierrot-Deseilligny 2012)*; (2) the need to prevent osteoporosis, attenuate infections, as well as to improve non classical clinical outcomes *(Pierrot-Deseilligny 2013)* in patients with marked vitamin D deficiency; (3) the safety of a moderate supplementation in terms of hypercalcemia and other significant adverse events *(Pierrot-Deseilligny 2012, Hathcock 2007)*; (4) the control of inflammatory components of the disease. In temperate countries, the supplementation should never be stopped since there is no durable storage of this vitamin in the organism. However, analysis of vitamin D and its metabolites should be made prior to supplementation so as to monitor responsiveness to treatment at preset times after application *(Luque-Cordoba 2017)*.

Parkinson's Disease and Vitamin D

Parkinson's disease (PD) is a progressive neurodegenerative disease characterized by slow, selective dopaminergic neuronal loss. Symptoms include dyskinesia, rigidity, and tremor, as well as postural instability and gait disorders *(Bonnet 1999)*. Like for MS, vitamin D could play a key role in neurological disorders such as PD and dementia *(Eyles 2005, Prufer 1999)*.

In the context of PD, potential neuroprotective effects exerted by vitamin D include the notion that 1,25(OH)2D indirectly inhibits the synthesis of nitric oxide, a free radical that can damage cells *(Garcion 2002)*; secondly, it indirectly stimulates the synthesis of the antioxidant glutathione *(Garcion 2002)*, and, thirdly, vitamin D may act as a neurotrophic factor, through the stimulation of nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF) and neurotrophin 3 (NT3) *(Naveilhan 1996, Musiol 1997, Neveu 1994)*.

Cross-sectional studies have linked vitamin D deficiency and PD incidence *(Evatt 2008, Sato 2005)*. The first longitudinal study investigating the association between vitamin D status and risk of PD showed that low serum vitamin D levels predicted an elevated risk of PD *(Knekt 2010)*, but such findings were not reproduced in a study published later *(Shrestha 2016)*. A recent study by Fullard et al. evaluated vitamin D levels in a population at risk for developing PD, derived from The Parkinson Associated Risk Syndrome (PARS) study cohort. In particular, they found that vitamin D levels did not differ in the high-risk group when compared with age- and sex-matched controls, suggesting that sustained vitamin D insufficiency is not common before a diagnosis of PD *(Fullard 2017)*. This is in line with another recent study of Larsson et al., which used Mendelian randomization approach to minimize confounding effects and prevent bias because of reverse causation. According to recent findings, their results showed no association between genetically-predicted lower vitamin D concentration and PD *(Larsson 2017)*.

In patients with overt PD, however, it has been repeatedly demonstrated that serum 25(OH)D is significantly lower than in healthy controls *(Sato 2005, Topal 2010, Sato 1997, Abou-Raya 2009, Evatt 2011, Senel 2011, Van den Bos 2013)*, and serum 25(OH)D levels progressively decrease with increasing severity of PD (*Sato 1997, Rimmelzwaan 2016, Ding 2013)*. Intuitively, this link could relate to the reduced sun exposure, hence the

dermal synthesis of vitamin D, in patients with progressive motor limitations, whereas no significant difference in vitamin D intake was found in patients versus controls *(Miyake 2011)*. When the relationship between serum 25(OH)D level and functional scores such as balance is accounted for, a study by Peterson et al. investigated this association using five tests (i.e., the motor control test, sensory organization test, sit and stand test, walk and turn test, and the unilateral stance eyes open and closed test) in 40 PD patients and showed a significant positive correlation between serum 25(OH)D and automatic postural responses *(Peterson 2013)*.

Interventional studies examined the correlation between vitamin D supplementation and PD outcomes. In particular, Suzuki and colleagues *(Suzuki 2013)* examined the impact of vitamin D supplementation (1200 IU per day for one year) on disease progression using various PD-related outcomes measured with the modified Hoehn & Yahr (H&Y) scale and the Unified Parkinson Disease Rating Scale (UPDRS). In a double-blind placebo-controlled trial on 104 patients with PD studied for 12 months, 56 PD patients received 1200 IU vitamin D per day and 58 received placebo. The authors found that those on placebo experienced worsening PD outcomes, while the deterioration was significantly milder in the vitamin D-supplemented group. Analysis of the VDR single nucleotide polymorphisms (SNPs) in these patients found that those bearing the FokI VDR genotype (rs10735810) TT allele had a more significant and consistent response to vitamin D supplementation than individuals with VDR FokI CT, while patients with FokI CC showed no significant effect of vitamin D supplementation compared to placebo. Alternatively, the effects of vitamin D were not influenced by other VDR variants, that is, rs1544410 (BsmI), rs731236 (TaqI), rs7975232 (ApaI), and rs11568820 (Cdx2) *(Suzuki 2013)*. The role of other VDR polymorphisms has also been investigated in PD *(Peterson 2013, Lin 2014, Han 2012, Torok 2013, Suzuki 2012, Tanaka 2017)*. The rs10735810 (FokI) C allele was present at significantly higher frequency in PD patients as compared to controls *(Han 2012, Torok 2013)*. In a study by Suzuki et al. in a Japanese population, the VDR variant FokI CC was associated with milder forms of PD *(Suzuki 2012)*. Tanaka et al. found a significant inverse association between VDR SNP rs2228570 and the risk of PD, but this fell below significance after adjustment for multiple comparisons *(Tanaka 2017)*.

Based on the previous, additional studies are warranted to elucidate the effects of vitamin D on neuroprotection in PD.

Alzheimer's Disease and Vitamin D

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive and irreversible cognitive deficits and behavioural alterations. Memory impairment and loss of spatial memory are hallmarks of the disease, and lead to complete incapacity and death within three to nine years since diagnosis *(Querfurth 2010)*. At pathology, AD presents with amyloid-β (Aβ)-rich plaques, neurofibrillary tangles (NFTs), synapse loss, and atrophy in brain areas associated with memory and executive functions *(Jack 2005, Mosconi 2005, Selkoe 2001, Sperling 2011)*. Aging is a strong risk factor for AD *(Perluigi 2014, Sultana 2013)*. Other major risk factors for AD include gender, family history, genetics, and head trauma, low educational attainment and environmental factors. There is accumulating evidence suggesting a significant association between vitamin D and AD *(Keeney 2015, Moretti 2017, Patel 2017, Littlejohns 2014)*. In vitro, vitamin D stimulates macrophages which increase the clearance of Aβ plaques *(Masoumi 2009, Mizwicki 2012)*, reduces amyloid-induced cytotoxicity and apoptosis in primary cortical neurons *(Dursun 2011)*, and influences Aβ stimulation of induced nitric oxide synthase (iNOS), which contributes to modulate the inflammatory process related to AD *(Dursun 2013)*. Recent genome-wide association studies have focused on the role of VDR polymorphism in late onset AD (LOAD) susceptibility *(Gezen-Ak 2012)*. A decreased level of VDR mRNA has been reported in hippocampal region by analyzing postmortem AD brain *(Sutherland 1992)*. Alterations in VDR and 1,25-MARRS genes related to the action and metabolism of vitamin D result in the inefficient utilization of vitamin D, making neurons vulnerable to neurodegenerative changes *(Dursun 2011, 2013, Gezen-Ak 2007, 2012, 2013)*. Associations have been found between AD, VDR gene polymorphisms and megalin to support this inference *(Dursun 2013, Gezen-Ak 2007, 2011, 2012, 2013, Beydoun 2012)*. Recently, Gezen-Ak et al. focused on the creation of a condition that prevents the genomic or non-genomic actions of vitamin D by individually or collectively silencing the VDRs (VDR or protein disulfide isomerase A3 (PDIA3)/1,25-MARRS) in primary cortical neurons *(Gezen-Ak 2011)*. Using this experimental model, an effect of VDRs disruption could be documented on proteins involved in secretases relating to amyloid pathology and Aβ 1−42 production *(Gezen-Ak 2017)*. The authors then suggested that vitamin D and its receptors VDR and PDIA3 could play important roles in amyloid processing pathway

within neurons *(Gezen-Ak 2017)*. The vitamin D carrier, DBP, was also identified at decreased levels upon plasma protein profiling of subjects with mild cognitive impairment and in AD patients, when compared to healthy controls *(Muenchhoff 2015)*. In a study by Moon et al., the DBP was found to inhibit Aβ aggregation and prevent Aβ mediated cell death in cultured hippocampal cells *(Moon 2013)*. Both monomeric and oligomeric Aβ bound to DBP in a dose-dependent manner. Following addition of Aβ, DBP treatment resulted in reduced synapse loss in mouse hippocampus and rescued Aβ-induced memory deficits *(Moon 2013)*. Collectively, these results suggest that impairment in vitamin D transport is present even at early stages of AD, namely, prior to development of dementia. Based on this evidence, it is feasible to hypothesize that AD might be the consequence of a hormonal imbalance in which the critical hormone is vitamin D. Vitamin D promotes neuroprotection in AD via regulating nerve growth factor and neurotransmitters *(Brown 2003, Veenstra 1998)*, increasing the amyloid metabolism *(Durk 2014, Ito 2011)*, imposing an anti-inflammatory action *(Baeke 2010, Krishnan 2010)* and promoting calcium homeostasis *(Brewer 2001)*. With this emerging evidence, vitamin D has important roles as a neurosteroid in AD.

Epidemiological studies outlined a strong correlation between deficiency of vitamin D and neurodegeneration associated with AD. As many as 70–90% AD patients are vitamin D deficient, and AD patients are the most vulnerable group to develop neurodegenerative disorder due to additive effect of Vitamin D deficiency along with aging factor *(Durk 2014, Annweiler 2011, Bischoff-Ferrari 2012, Holick 2007)*. Although vitamin D status is a crucial but non-specific risk factor for AD *(Annweiler 2015)*, Cui et al. suggested that specific "critical windows" may exist during which vitamin D deficiency might result in the most detrimental brain outcomes. During these timeframes, vitamin D supplementation could be the most beneficial factor to prevent long-term damage to the brain *(Cui 2015)*. A potential therapeutic window during which vitamin D might provide benefits to reduce the risk, or delay the onset, of AD could occur during the pre-clinical and mild cognitive impairment ages, when measurable changes in glucose utilization and Aβ accumulation already occur *(Etgen 2011)*.

Llewellyn et al. showed an increased risk of losing points on the Mini-Mental State Examination (MMSE) in six years in 175 older adults with severe 25(OH)D deficiency (<10 ng/mL) compared to 157 subjects with sufficient vitamin D (>30 ng/mL) *(Annweiler 2011,*

Llewellyn 2010). Slinin et al. followed up the association between lower 25(OH)D levels and cognitive decline in aged individuals (>65 years) for four years *(Annweiler 2011, Slinin 2010)*. A meta-analysis by Etgen et al. highlighted an increased risk of cognitive impairment in patients with vitamin D deficiency *(Etgen 2012)*. Balion et al. compared mean MMSE scores with levels of 25(OH)D and showed higher average MMSE scores in those with higher 25(OH)D concentrations *(Balion 2012)*. Further, in the Chianti study, a large prospective study on 858 adults, cognitive decline was associated with lower concentrations of vitamin D, when observed over a period of six years *(Llewellyn 2010)*. The association between low vitamin D and increased risk of AD has been confirmed in other long-term studies *(Annweiler 2012)*, and over 50% of published prospective studies showed an elevated risk of cognitive impairment after four to seven years of follow-up in participants with lower 25(OH)D levels compared with participants with higher 25(OH)D levels *(Annweiler 2015)*. In parallel, an increased incidence of vitamin D deficiency has been documented in AD patients *(Annweiler 2011, Balion 2012)*, while a seven-year follow-up study by Annweiler et al. found that higher dietary intake of vitamin D was associated with a lower risk of developing AD in older women *(Annweiler 2012)*. Together, these studies suggested that low vitamin D poses a serious risk for AD development and progression.

Interventional studies using vitamin D in combination with anti-AD drugs have shown fairly encouraging results. A recent six-month trial study by Annweiler et al. observed that the combination of memantine, a multi-target therapy for AD-related dementias (ADRD), and vitamin D elicited a superior effect than memantine or vitamin D alone in halting the cognitive decline among AD patients tested by MMSE *(Annweiler 2012)*. The AD-IDEA trial *(Annweiler 2011)*, a randomized placebo-controlled trial initiated in 2011 and completed in 2016, further investigated the effect of this combination therapy and awaits publication *(Annweiler 2011)*. Fiala and Mizwicki *(Fiala 2011)* added further evidence that combined administration of vitamin D and docosahexaenoic acid (DHA) could enhance direct effects and immune protection of neurons against brain amyloidosis and other brain insults. As vitamin D targets various pathological processes of ADRDs, it may thus increase the effectiveness of standard anti-dementia treatments, or improve at least partially the resistance to these treatments.

Long-term randomized clinical trials of vitamin D supplements in large populations of middle-aged adults from different countries are warranted to study the conversion rate to mild cognitive impairment or AD outcome, with the most obvious question remaining whether optimizing vitamin D status will reduce the risk AD or be of therapeutic benefit following the onset of the disease.

Neurocognitive Disease and Vitamin D

Epidemiological studies outlined an association between 25(OH)D and parameters of cognitive function, such as memory, orientation and executive functions *(Llewellyn 2009, Buell 2009, Lee 2009)*. Hypovitaminosis D is associated with altered domain-specific cognitive and executive functions, particularly information processing speed, mental shifting and working memory *(Balion 2012)*, whereas the effect on episodic memory is milder *(Balion 2012)*. These functions are required for the cognitive control of behavior and the execution of cognitive programs in real time, and are involved in high-level motor control *(Beauchet 2012)*. In older adults living independently, low serum 25(OH)D concentrations were shown to be significantly associated with cognitive impairment *(Peterson 2012)*. In addition, certain variants of human VDR gene are associate with increased risk of cognitive decline *(Sutherland 1992, Gezen-Ak 2007)*. A study by Llewellyn et al. in 1766 older adults from the Health Survey for England 2000 described an inverse association between 25(OH)D levels and cognitive impairment *(Llewellyn 2009)*, and patients with 25(OH) levels <20 ng/mL harboured a 230% higher risk of cognitive impairment when compared with those having 25(OH)D levels >20 ng/mL. The same group of authors showed that impairment of cognitive function during a three- and six-year follow-up was greater in patients who were initially vitamin D deficient *(Llewellyn 2010)*. In fact, cognition scores measured by MMSE and Trail-Making Tests A and B were poorer in vitamin D-deficient subjects as compared to those whose vitamin D levels were sufficient. Having severe vitamin D deficit at baseline increased the likelihood of showing cognitive decline of executive functioning (measured by MMSE and Trail B) but not attention (measured by Trail A) at the six-year follow-up *(Llewellyn 2010)*. Similar results have been obtained in a systematic review and meta-analysis *(Balion 2012)*. Full evidence of a causal relationship between low vitamin D and cognitive impairment is far to be

demonstrated and the existence of this association has even been challenged in other studies *(Slinin 2010, Balion 2012, McGrath 2007, Annweiler 2010)*. Nevertheless, some authors concluded that vitamin D deficiency in older adults is associated with dementia and that vitamin D supplementation might have a protective effect (Annweiler 2011, 2012, 2013, Buell 2010, Barnard 2010). Importantly, systematic reviews and meta-analyses carry several limitations while cross-sectional studies cannot provide causal links, that is, answer the question of whether vitamin D deficiency leads to cognitive decline or whether people with a cognition disorder have lower exposure to sunlight and/or lower vitamin D intake *(Van der Schaft 2013)*. A recent systematic review by Sommer et al. evaluated the influence of vitamin D deficiency on dementia risk using longitudinal studies *(Sommer 2017)*, and concluded that available findings were consistent with the hypothesis that low vitamin D levels could contribute to the development of dementia, although methodological issues should be taken in account, such as residual confounders, single vitamin D assessments, and sunlight exposure *(Sommer 2017)*.

Based on these potential links, it appears advisable that normal levels of vitamin D should be attained to confer potential protection against the risk and progression of AD. In older adults and AD patients, dietary vitamin D intake was associated with better cognitive performance *(Annweiler 2010)*, and being in the highest quintile of vitamin D dietary intakes was associated with a lower risk of AD after seven years, when compared with the lower four quintiles combined (adjusted OR = 0.23; p = 0.007) *(Annweiler 2012)*. This pro-cognition effect was confirmed in before–after and non-randomized clinical trials on vitamin D supplementation, where improved cognitive performance was recorded in the older population as well as AD patients *(Przybelski 2008, Annweiler 2012, Stein 2011)*. Short-term studies underscored the cognitive benefits elicited by four-week vitamin D supplementation *(Przybelski 2008)*, with marked improvements in executive functions and information processing speed *(Annweiler 2013)*. The administration of supra-physiological doses, such as 7000 IU/day, seems to provide no additional benefit *(Stein 2011)*, while common supplementation dosages around 800–1200 IU/day appear to be sufficient and desirable *(Annweiler 2013)*. Because confounding factors could influence cognition outcomes *(Annweiler 2015)*, it remains crucial to conduct appropriately designed, randomized, placebo-controlled interventional trials to test the effectiveness of, and responsiveness to, vitamin D supplementation in AD, once the basal level of

vitamin D and other possible confounders are appropriately accounted for *(Annweiler 2016)*.

Amyotrophic Lateral Sclerosis and Vitamin D

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease of the upper and lower human motor system, linked to abnormalities in the glutamate neurotransmitter system *(Kiernan 2011)*. With familial ALS accounting for 5–10% of cases, neurodegeneration is a hallmark of ALS and results from the complex interaction between genetic and molecular pathways, encompassing glutamate excitoxicity, generation of free radicals, cytoplasmic protein aggregates, modifications of the superoxide dismutase (SOD1) enzymes activity, mitochondrial dysfunction, accumulation of intracellular calcium, all leading to disruption of axonal transport through accumulation of intracellular aggregates *(Vucic 2009, Pasinelli 2006, Neusch 2007, Bruijn 1997, 1998)*. Challenging the "neurocentric" view of ALS, recent evidence suggests that non-neural cells such as microglia, astrocytes, peripheral blood mononuclear cells (PBMCs) and skeletal muscle fibers may partake in motor neuron degeneration and cooperate to exacerbate ALS *(Pansarasa 2014)*. Genetic studies have identified several proteins linking vitamin D to ALS pathology: MHC class II molecules; toll-like receptors; poly(ADP-ribose) polymerase-1 and calcium-binding proteins *(Long 2013)*. Genome-wide analysis further identified a number of biologically relevant candidate genes with VDR-binding sites within or in close proximity *(DeLuca 2013)*, and it has been shown that candidate genes involved in gene transcription associated with ALS signaling are modulated by 1,25(OH)2D *(Tuohimaa 2013)*. Altered calcium homeostasis appears to contribute significantly to selective neuronal injury in ALS, with the putative cause of vulnerability being the low levels of the calcium-binding proteins calbindin-D28K and of parvalbumin, which can be elevated by gene therapy and by vitamin D supplementation *(Long 2013, Karam 2013, Gianforcaro 2013)*. Calbindin-D28K is a 1,25(OH)2D-induced calcium-binding protein, and is stimulated at the protein and gene level by calcitriol in human syncytiotrophoblast cells *(Halhali 2010)*. Parvalbumin increases in the caudate putamen of rats with vitamin D hypervitaminosis *(De Viragh 1989)*, suggesting a link between parvalbumin metabolism in the caudate putamen and vitamin D variations in the bloodstream. Also, the injection of

80–120 ng calcitriol in the cerebral ventricles of adult rats induced positive immunoreactivity for calcium binding proteins in ventral motor neurons *(Alexianu 1998)*, implying a potential modulatory effect of calcitriol on the expression of calcium binding proteins in the motor system. In mice expressing ALS-linked mutated SOD1, an animal model of ALS, vitamin D intake increased muscle strength with no significant effect on lifespan *(Gianforcaro 2013)*, whereas vitamin D3-deficient diet delayed disease onset but decreased motor performance *(Solomon 2011)*. Treatment of rodent motor neurons with 1,25(OH)2D promoted efficient binding and nuclear translocation of the VDR, exerted a neuroprotective effect against the motor neuron-restricted Fas death pathway, and acted by potentiating the trophic activity of neurotrophins *(Camu 2014)*.

Among the other effects of vitamin D on the proteome linked to ALS, vitamin D has been shown to upregulate VEGF by response element in the VEGF promoter, which may delay progression of ALS *(Cardus 2009)*. In addition, vitamin D increases neurotrophic factors *(Oppenheim 1996)*, and acts to promote the effectiveness of IGF-I by increasing expression of IGF-I receptors *(Gomez 2006)*, which promotes survival and motor function parameters in SOD1 animals *(Lepore 2007)*. Further, vitamin D exerts pro-differentiative, immunomodulatory and anti-inflammatory properties (e.g., by reducing tumor necrosis factor (TNF)-α, IL-1B, and cyclooxygenase (COX)-2), which could prove useful to control the expression of pro-inflammatory molecules linked to ALS progression *(Papadimitriou 2010, Szodoray 2008)*, as well as to partake in autophagy-based misfolded SOD1 aggregates clearance *(Von Essen 2010)*. A direct association has also been demonstrated between circulating parathyroid hormone (PTH), which increases in parallel with the severity of vitamin D deficiency, and the duration of ALS in males *(Yanagihara 1984)*. Finally, the gene encoding DBP (group-specific component, Gc) is a key factor for regulating calcium homeostasis through the vitamin D endocrine system. As such, a proteomic study of Portuguese patients with familial ALS (FALS), not carrying SOD1 mutations, isolated an isoform of DBP that was identified as GC2, which was absent in healthy controls, and noted a decrease of more acidic isoforms of DBP in FALS; collectively, these results suggest that GC2 polymorphism of DBP could constitute a risk factor for ALS *(Palma 2008)*.

An interaction also links vitamin D to muscle morphology and strength *(Bischoff-Ferrari 2012)*. The VDR is expressed in skeletal muscle and modulates 25(OH)D uptake in

myofibers *(Girgis 2014)* via the cell membrane receptor megalin, and DBP is retained in these cells by its specific binding to actin *(Abboud 2013)*. Silencing of the VDR in mice is associated with smaller muscle fibres and persistent immature muscle-gene expression during adult life *(Endo 2003)*. In C2C12 muscle cells, 25(OH)D and 1,25(OH)2D dose-dependently exert an auto-regulatory loop to regulate the expression of genes related to proliferation, differentiation, and myotube size *(Girgis 2014)*. Tracer studies and epidemiological investigations have described a relationship between 25(OH)D levels and parameters relating to muscle, such as lean body mass or exercise performance, leading to speculate that skeletal muscle cells could act as a reservoir of 25(OH)D capable of protecting it from liver uptake, degradation and excretion *(Abboud 2013)*. In their meta-analysis, Stockton et al. suggested that the beneficial effects of vitamin D on lower extremity muscle strength depended on baseline 25(OH)D levels <25 nmol/L, suggesting that vitamin D effects on muscle performance are more evident in individuals with lower initial 25(OH)D levels *(Stockton 2011)*. Therefore, the association between vitamin D and muscle morphology and function could lead to hypothesize that vitamin D effects on the muscle contribute to ALS-related function scores *(Paganoni 2017)*.

These mechanistic associations notwithstanding, conflicting results from clinical studies exist on prevalence rates of vitamin D deficiency and the effect of vitamin D supplementation in ALS. Using mortality data, a geographic distribution of ALS with a northwest to southeast gradient was demonstrated in an American cohort *(Sejvar 2005)*, a finding recently mirrored in a study showing higher ALS-associated death rates in more northern states *(Uccelli 2007)*. An earlier study suggested a high prevalence of hypovitaminosis D in ALS patients *(Sato 1997)*. A subsequent study investigating vitamin D levels in 37 consecutive ALS patients found that 81% had vitamin levels below the normal range *(Karam 2013)*. In ALS patients, severe vitamin D deficiency was associated with four-fold increased rate of functional decline and significantly reduced survival expectancy *(Camu 2014)*. In this study, there was an association between low vitamin D and a survival time in ALS patients, even after excluding non-ambulatory patients with vitamin D deficiency. In 71 sporadic ALS patients, 1,25(OH)2D levels were found significantly lower than in controls, with positive correlations between 1,25(OH)2D and both ALS functional rating scale (ALSFRS-R) and Manual Muscle Test scoring (MMT) *(Cortese 2015)*. Moreover, the levels of 1,25(OH)2D levels were lower in spinal-onset ALS

compared to bulbar-onset ALS, and only in spinal-onset ALS 1,25(OH)2D levels positively correlated with functional scores and negatively with disease duration, suggesting that different impairment of vitamin D signaling pathways in cortical/spinal cord and bulbar motoneurons could explain different levels related to ALS site of symptoms onset *(Cortese 2017)*. However, divergent conclusions were reached by other studies. In a small retrospective study in ALS and controls, lower than expected rates of vitamin D deficiency were observed in ALS, and there was a lack of relationship between vitamin D and clinical variables related to ALS *(Libonati 2017)*. A prospective study evaluating vitamin D levels in 106 ALS patients, half of whom were on riluzole, found low vitamin D levels in 69% of cases (<30 ng/mL), with 50% falling into the category of "insufficient" 25(OH)D and 19% being "deficient" *(Paganoni 2017)*. In this cohort, higher vitamin D was associated with higher concurrent gross motor ALSFRS-R scores at baseline. However, vitamin D failed to predict the future rate of disease progression, suggesting that low vitamin D may be a result rather than a cause of worse health in people with ALS, perhaps due to poor mobility and/or metabolic confounders *(Paganoni 2017)*. In a study focused on the association of clinical disease outcome with bone metabolism, including serum 25(OH)D concentrations and bone mineral density, Yang et al. found that 34% of Korean ALS patients were severely vitamin D deficient (<10 ng/mL) and 81% were vitamin D deficient (<20 ng/mL); nevertheless, 25(OH)D concentration failed to predict survival, and older age at onset and bulbar onset were the most predictive factors for survival outcome *(Yang 2016)*. Emphasizing these contrasting findings even further, Blasco et al. reported that high-range vitamin D levels were correlated with a worse rather than with the improved prognosis in ALS patients *(Blasco 2015)*.

The effect of vitamin D supplementation on ALS progression has only been investigated in a few studies to date. Daily supplementation with 2000 IU of vitamin D for nine months to ALS patients improved the revised ALSFRS-R scores, such that 20 of these patients received vitamin D and their functional scores declined less rapidly than those patients who did not receive supplements *(Karam 2013)*. Inversely, in their retrospective study Libonati et al. reported that cholecalciferol treatment for three and six months (loading dose of 100,000 IU every week for four weeks, then maintenance dose of 25,000 IU every 15 days) did not improve clinical parameters compared to untreated ALS patients *(Libonati 2017)*.

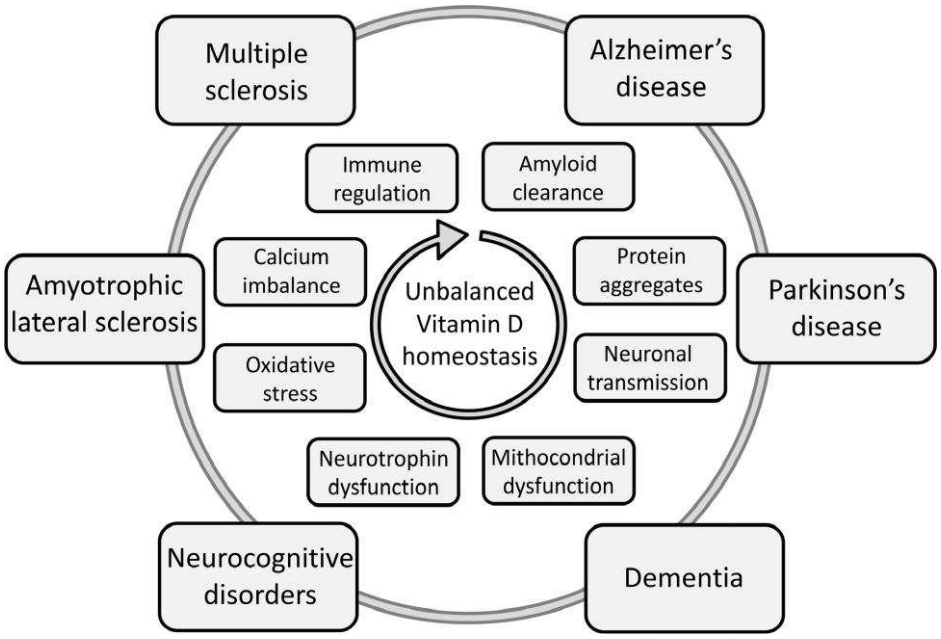
Based on these results, evidence to support a causative role for low vitamin D in ALS is weak, and low levels of vitamin D found in patients with ALS could depend on limited mobility, hence sun exposure, in ALS patients. The benefits of vitamin D supplementation in ALS patients remain to be elucidated.

Conclusions

Accumulating evidence suggests that vitamin D acts like a neurosteroid (Groves 2014) and is required for normal brain development and function *(Garcion 2002, Balden 2012, Eyles 2005, Kalueff 2007, Cui 2010, 2013, Kesby 2010, Groves 2014, McCann 2008)*. The association between low levels of 25(OH)D and a wide spectrum of neurodegenerative conditions such as multiple sclerosis, Alzheimer's disease, Parkinsons's disease and neurocognitive disorders, is supported by in vitro and in vivo data. Less convincing, on the other hand, appears to be the causative link between vitamin D deficiency and the onset, progression and clinical burden of amyotrophic lateral sclerosis. Regrettably, most studies conducted to date have not controlled for reverse causality, that is, low levels of vitamin D possibly being due to impaired mobility or sun-avoidance behavior. As such, there is a need for randomized clinical trials on vitamin D treatment in patients at risk of neurodegenerative disorders so as to optimize knowledge and precision on the efficiency of vitamin D congeners, appropriate dosing, and correct biochemical and clinical monitoring. A proper endocrine approach would suggest the use of vitamin D congeners related to optimal drug delivery through the brain blood barrier *(Hajiluian 2017)*, using gradually increasing doses to avoid the risk of falls in frail populations *(Bischoff-Ferrari 2016)*. In view of the advantage that vitamin D supplementation is readily available and affordable, there is a need for further research in this field.

Figures

Figure 1. Schematic overview of neurological disorders and their underlying mechanisms relating to impaired homeostasis of the vitamin D system.



CONCLUSIONS

The studies reported in this thesis described the complex phenotypes and metabolic features of severe obesity and thyroid disorders, conveying to the study of the crosstalk between obesity and thyroid function.

In particular, the data reported in chapter one depict the wide range of metabolic features and complications underlying the condition of severe obesity, as well as the effects of treatments used for obesity-related comorbidities (i.e., type 2 diabetes mellitus, dyslipidaemia) in modifying the metabolic pattern of these patients.

The results illustrated in chapter 2 underline the fundamental role of metabolic mileau and inflammation related to insulin resistance in predisposing patients to the development of thyroid disorders and, in particular, thyroid cancer. In fact, the role of adipokines and insulin resistance as metabolic biomarkers in thyroid tumorigenesis appears very interesting due to their potential involvement in cell proliferation. Moreover, with the complexity of IR-related pathways, an array of molecules could represent novel therapeutic targets in health and disease.

Chapter 3 reported new data on the attractive association between obesity and thyroid function. In particular, we demonstrated that fT4 levels appeared to be related to the overall metabolic phenotype of obese patients and that a short-term weight loss seems capable of reactivating the favorable relationship between REE and thyroid hormones in severe obesity, via interactions with fat-free mass. Moreover, our data showed that obesity has complex effects on the metabolic outcome of hypothyroid patients in replacement therapy, who required lower L-T4 doses compared to normal weight subjects.

Finally, it is important to underline that the increased prevalence of obesity worldwide may further confound the definition of the normal TSH range in population studies. More research is necessary to investigate these aspects and to define the normal range of TSH in adult obese subjects.

FUTURE PERSPECTIVES

ANGPTL8 and Irisin: new hormones involved in obesity and metabolism

Background. The recently described myokine and adipokine, irisin has been proposed to be secreted upon exercise to promote the browning of beige fat cells in white adipose tissue that results in enhanced thermogenesis and increased energy expenditure. Initial studies suggested a potential role of irisin as treatment option for obesity and associated diseases such as type 2 diabetes mellitus (T2D) and stimulated further research.
Betatrophin is a novel protein predominantly expressed in human liver. Increasing evidence has revealed an association between betatrophin expression and serum lipid profiles, particularly in patients with obesity or diabetes. However, its precise physiological role remains unclear at present.

Aim of the study. The aim of this study is to investigate circulating irisin and ANGPTL8 concentrations in obese patients stratified as metabolically healthy and unhealthy in comparison with lean subjects, in order to characterize their role in glyco-lipid metabolism and insulin resistance, hence their potential involvement in multiorgan damage process linked to hyperglycaemia of obesity.

Methods. Due to recent controversies about circulating irisin including difficulties of its specific detection in human serum, we decided to evaluate firstly the expression of irisin by Western Blot. First of all, we detected irisin in serum using a specific antibody, that also is used in ELISA assay. The major problem faced in the analysis of human serum is the broad range of its protein constituents. So we decided to deplete the serum with albumin/IgG depletion kit and further analyzed depleted serum in Western immunoblot. In particular with Western Blot we immunodected irisin in human muscle protein, to confirm the real presence in muscle and in human serum. We detected a strong band around 25 kDa in these sample and also using recombinant irisin, to confirm the specificity of the antibody.

We enrolled 12 metabolically healthy (MHO) and 12 metabolically unhealthy obese subjects (MUO) with type 2 diabetes. These MHO and MUO subjects were comparable for anthropometric and body composition data. Biochemical parameters included glucose homestasis, lipid panel, adiponectin, leptin, and clinical analyses targeting organ damage. Betatrophin and irisin were analyzed using by ELISA. Patients enrolment for the 2 groups and lean controls is ongoing.

Preliminary results. Circulating concentrations of irisin were higher in MUO than MHO, probably because irisin may act as an adaptive response to decrease insulin resistance. Circulating levels of betatrophin were significantly lower in MUO than MHO. The decreased levels of betatrophin would seem negatively related to hyperglycemia and to the degree of insulin resistance. Because of betatrophin inhibits LPL, and thereby suppresses triglyceride clearance and increases serum triglycerides, the decreased serum betatrophin levels in diabetes may play a role in explaining the hypertriglyceridemia in DMT2.

Future perspectives. Previous studies provided controversial findings on serum irisin and betatrophin. We will evaluate their involvement in the development and progression of obesity-associated type 2 diabetes, and study the relation with parameters of adiposity, muscle mass and adipokines, organ damage, and the effect of short-term weight loss. We are currently extending research on irisin and betatrophin actions in obese adult patients with Prader Willi Syndrome.

Pilot study on metabolomic profiling of metabolically healthy and metabolically unhealthy obesity

Background. While obesity is conventionally deemed as a risk factor for the development of the metabolic syndrome and its associated comorbidities, it is now recognized that individuals in the same BMI category can have substantial heterogeneity of metabolic features. Recent interest has focused on a unique subgroup of obese individuals described as “metabolically healthy obesity” (MHO), who have normal metabolic features despite their increased adiposity. This trait has attracted growing interest in relation to the profiling of clinical determinants and long-term outcomes. Attention has recently focused on the metabolome as a tool to screen the complete metabolite composition of a system, such as a cell or an organism. In obesity, studies on the metabolome illustrated the chemical changes associated with adiposity and regional fat accumulation involving: abnormal metabolism of branched-chain and aromatic amino acids; fatty acid synthesis and oxidation, as well as serum phospholipids and their degree of saturation; compounds involved in carbohydrate metabolism such as fructose, glycerol and mannose; and compounds involved in nucleotide metabolism such as uridine. Evidence is also emerging from the study of the volatilome, the volatile chemical signature from individuals that is capable of providing invaluable information on the physiological status, can contribute to expand current knowledge on obesity traits and their trajectories. So far, several metabolites and volatiles associated with obesity have been thus identified and confirmed to be disturbed extensively in humans. It would be therefore of interest to differentiate the metabolomic profile and volatilome of MHO against “metabolically unhealthy obesity” MUHO to detail how these traits differ or overlap.

Aim of the study. This is a study performed in collaboration with university of Turin and university of Nebraska. We aim to explore the metabolic fingerprint of obese patients stratified as metabolically healthy and unhealthy individuals. An effort will be made to obtain abdominal sc fat tissue biopsies.

Patients and Methods. Following a preliminary bio-anthopometric screening of a larger group of consecutive patients with severe obesity (BMI >40 kg/m2) admitted to our tertiary care center, 50 obese men will be enrolled and subdivided into equal groups of MHO and MUHO subjects. MHO subjects will be defined by normal values for all three risk factors. Individuals crossing the risk threshold for all three factors will meet ATPIII criteria for MUHO. Obesity is defined according to BMI status >30 kg/m2. A group of healthy lean subjects will be made available as control for subsequent comparative analyses. Obese study participants will undergo full-scale metabolic assessment at baseline incorporating: Biochemical analysis, urine analysis, 2h 5-point OGTT with determination of glucose, insulin and c-peptide, determination of leptin, adiponectin oligomers, irisin and lipasin (ELISA), body composition, indirect calorimetry for determination of REE and RQ. At the end of a 4 week diet plus exercise inpatient program, the previous assessment will be repeated with the exception of the OGTT. Both at study entry and end, participants will undergo withdrawal of plasma samples and 24h urine collection for the study of metabolome and volatilome, which will comprise low molecular-weight compounds that serve as substrates and products in metabolic pathways.

Expected results. Based on the current analysis, future research will concentrate on identifying distinct patterns of metabolome and volatilome expression between the group of MUO and MUHO, to generate individual fingerprinting, which will be clustered according to individual clinical outcomes. Differences in individual fingerprints will be mapped to pathway graphs for better understanding of the parts of the pathway that are altered due to clinical health.

Molecular characterization and biological behaviour of thyroid microcarcinomas

Background. The risk of loco-regional relapse and distant metastases of thyroid microcarcinomas (mPTC) is less than 2.5% and 0.3%, respectively. Neverthless, BRAF and TERT mutations, described in about 36% and 5% of mPTC respectively, seem to correlate with histopathological features of aggressiveness and higher risk of relapse. Among mPTC followed-up in our Department, we found a more aggressive behaviour with respect to literature, with relapse in 9.8% of cases, mainly loco-regional.

Aim of the study. Our study aims are to evaluate the molecular and inflammatory profile of mPTCs in our population, and to correlate this profile with the clinical and hysto-pathological characteristics.

Methods. We retrospectively evaluate the cases of mPTCs followed in the Department of Endocrinology of the "Maggiore della Carità" University Hospital in Novara from 2000 to 2015. Preliminarily, we performed the molecular characterization on tissues of 49 cases, analyzing the presence of mutations of BRAF, TERT, NRAS and RET/PTC; moreover, we correlated these findings with clinical, histological and follow-up data.

Preliminary results. In 32/49 cases we found one of the searched mutations; in particular, 29/49 carried mutated-BRAF (28/29 V600E, 1/29 K601E), 2/49 carried the RET/PTC translocation and 1/49 TERT mutation. The presence of V600E BRAF mutation positively correlated with higher tumour size ($p=0.03$); RET/PTC trans location correlated with the presence of capsular invasion ($p<0.05$), higher tumour size ($p<0.01$) and with tumour associated thyroiditis ($p=0.001$). No significant statistical correlation was found between mutations and tumour relapse.
Our preliminary data showed that the main mutation described in mPTCs were associated with aggressive histological characteristics, but they were not associated with higher risk of relapse, that appared more frequent in our population than in literature.

A larger cohorts and a wider molecular characterization will help to clarify the mutation associated with higher risk of malignancy and to recognize in a early stage those lesions needing for a more invasive treatment, avoiding the overtreatment of indolent lesions.

Thyroid function and obesity: a retrospective study in 5000 obese patients.

Background. Obesity is associated with many endocrine dysfunctions, including thyroid disorders. An increased interest on the relationship between thyroid function and obesity occurred. In particular thyroid hormones are involved in the regulation of basal metabolism and thermogenesis, playing an important role in lipid and glucose metabolism, fat oxidation and food intake. Although thyroid function is usually normal in obese patients, several studies have demonstrated that TSH and BMI are positively related, in particular TSH levels are slightly increased (usually below 10 IU/l) in obese patients compared to normal weight people. The increased TSH levels could be due to hormone resistance. In fact, in obesity T3 receptors are decreased as well as the negative feedback between TSH and the peripheral thyroid hormones, leading to increased levels of TSH and T3. On the contrary the increase of both TSH and T3 may be an adaptation process to increase REE and energy expenditure. To date, studies investigating the relationship between obesity, its anthropometric or metabolic features and TSH have been investigated in small population samples. Likewise, population studies have conventionally included only small severely obese samples.

Aim of the study. We aim to evaluate the relationship between anthropometric measures, biochemical parameters and serum thyroid hormones (TSH, fT4) concentrations in euthyroid obese subjects across the threshold of severe obesity, in order to characterize the modulators of thyroid function in condition of obesity.

Methods. We retrospectively enrolled 5000 obese subjects (BMI > 30 Kg/m2) referred to the Division of General Medicine of San Giuseppe Hospital, Piancavallo from 1999 to 2014. Patients' data were collected through retrospective medical records including gender, age, smoking status, biochemical analysis (TSH, fT4, glucose, insulin, AST, ALT, GGT and lipid panel), anthropometric data (weight, height, body mass index, waist and hip circumference, WHR), body composition parameters and calorimetric data. Patients with known thyroid disorders and/or under the use of levothyroxine or other medications that cause alterations in thyroid function were excluded.

Preliminary results. TSH levels in obese patients differ from those reported in the international literature for lean subjects (average TSH levels ranging between 1.49-1.79 µU/mL), with a serum TSH that seems higher in the obese (mean ± SD: 2.1±1.2 µU/mL)

In our obese population, the study of correlation coefficients showed that TSH levels were positively correlated with BMI ($r=0.07$, $p<0.0001$), fat mass ($r=0.11$, $p<0.0001$), total and HDL-cholesterol ($r=0.04$, $p<0.01$) and insulin levels ($r=0.03$, $p<0.05$), and negatively associated with age ($r=-0.05$, $p<0.001$), glucose levels ($r=-0.05$, $p<0.001$), lean mass ($r=-0.04$, $p<0.01$) and resting energy expenditure ($r=-0.05$, $p<0.001$).

FT4 levels were positively associated with glucose ($r=0.03$, $p<0.05$) and HDL-cholesterol ($r=0.07$, $p<0.0001$), whereas were negatively correlated with age ($r=-0.04$, $p<0.01$), BMI ($r=-0.05$, $p<0.001$), waist circumference ($r=-0.06$, $p<0.0001$), triglycerides ($r=-0.06$, $p<0.0001$) and lean mass ($r=-0.04$, $p<0.01$).

Several studies demonstrated that TSH levels are at the upper limit of the normal range or slightly increased in obese children, adolescents, and adults, and they are positively correlated with BMI *(Biondi 2010)*. TSH seems to be positively related to the degree of obesity. A positive correlation has been identified between serum leptin and serum TSH levels in obese individuals, which could reflect the positive association between TSH and BMI reported in some individuals. Leptin, adjusted for BMI, was found to correlate with TSH, which suggests that the increase in TSH and leptin levels in severe obesity could result from the increased amount of fat. Further statistical analyses are in progress.

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