CONTENTS

EDITORIAL
More news from IMPROVE-IT (IMProved Reduction of Outcomes: Vytorin Efficacy International Trial) ................................................................. 5
Niki Katsiki, Vasilios G. Athyros, Dimitris P. Mikhalidis

EXPERT CONSENSUS
Expert consensus on the rational clinical use of proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors ......................................................... 8

REVIEWS
Neoadjuvant therapy for advanced pancreatic neuroendocrine tumors: an emerging treatment modality? ................................................................. 15
Iraklis Perysinakis, Chrysanthi Aggeli, Gregory Kaltsas, George N. Zografos
Recent advances in the molecular mechanisms causing primary generalized glucocorticoid resistance ........................................................................ 23
Nicolas C. Nicolaides, Agaristi Lampropoulou, Amalia Sertedaki, Evangelia Charmandari

RESEARCH PAPERS
Lipid accumulation product is associated with metabolic syndrome in women with polycystic ovary syndrome ....................................................... 35
Djuro Macut, Ivana Božić Antić, Jelica Bjekić-Macut, Dimitrios Panidis, Konstantinos Tzimalos, Danijela Vojnović Milutinović, Olivera Stanoglović, Biljana Kastratović-Kotlica, Milan Petakov, Nataša Milić
Long-term follow-up results of growth hormone therapy for patients with adult growth hormone deficiency .................................................................... 45
Hidetoshi Ikeda, Masataka Kudo
Corticotropin-releasing factor (CRF) system localization of in human fetal heart ..................................................................................................... 54
Efterpi Chouridou, Maria Lambropoulou, Maria Kouretta, Christina Zarouchlioti, Ioanna Balgouranidou, Evangelia Nena, Nikolaos Papadopoulos, Ekaterini Chatzaki
Presence of the RET Cys634Tyr mutation and Gly691Ser functional polymorphism in Iranian families with multiple endocrine neoplasia type 2A ......................................................................................................................... 65
Maryam Nasiri Aghdam, Mohammad Reza Abbaspazegan, Alireza Tafazoli, Mohammad Aslzare, Zohreh Mosavi
The growth hormone axis and inflammatory responses after laparoscopic cholecystectomy .................................................................................. 73
Themistoklis Floros, Anastassios Philippou, Dimitrios Bardakostas, Dimitrios Mantas, Michael Koutrilis
Progestosterone pretreatment increases the stress response to social isolation in ewes ............................................................................................... 81
Aline Freitas-de-Melo, Juan Pablo Damián, Maria José Hötzel, Georgget Banchero, Rodolfo Ungerfeld
Prevalence and determinants of type 2 diabetes mellitus in a Greek adult population ............................................................................................. 88
Sofia Tzirona, Fotis Katsaras, Alexandra Bargiota, Stergios A. Polyzos, George Arapoglou, George N. Koukoulis
The relationship between retinol-binding protein 4 and apolipoprotein B-containing lipoproteins is attenuated in patients with very high serum triglycerides: A pilot study ......................................................................................................................... 99
Georgios A. Christou, Constantinos C. Tellis, Moses S. Eliaif, Alexandros D. Tslelpis, Dimitrios N. Kiortsis

CASE REPORTS
Clinical and biochemical responses after Gamma Knife surgery for a dopamine-secreting paraganglioma: case report ........................................ 106
Constantin Tuleasca, Yves Jaquet, Valerie Schweizer, Laura Negretti, Vera Magaddino, Philippe Maeder, Karim-Alexandre Abid, Benoit Lhermitte, Eric Grouzmann, Marc Levivier

Publishers: TECHNOGRAMMA: 380 Messogion Ave, 153 43 Athens, Tel.: +30 210 6000643, Fax: +30 210 6002295, e-mail: techn@hol.gr
Multiple endocrine neoplasia type 1 associated with a new germline Men1 mutation in a family with atypical tumor phenotype .... 113
Nikolaos Perakakis, Felix Flohr, Gian Kayser, Oliver Thomusch, Lydia Parsons, Franck Billmann, Ernst von Dobschuetz, Susanne Rondot, Jochen Seufert, Katharina Laubner

Intractable hypoglycaemia in a patient with advanced carcinoid syndrome successfully treated with hepatic embolization .......... 118
Angelos Kyriacou, Was Mansoor, Jeremy Lawrence, Peter J. Trainer

TSH-secreting pituitary adenomas treated by gamma knife radiosurgery: our case experience and a review of the literature .... 122
Zadalla Mouslech, Maria Somali, Anastasia Konstantina Sakali, Christos Savopoulos, George Mastorakos, Apostolos I. Hatzitolios

Identification of a novel mutation of the PRKAR1A gene in a patient with Carney complex with significant osteoporosis and recurrent fractures .......................................................................................................................................................................... 129
Labrini Papanastasiou, Stelios Fountoulakis, Nikos Voulgaris, Theodora Kounadi, Theodora Choreftaki, Akrivi Kostopoulos, George Zografos, Charalampos Lyssikatos, Constantine A. Stratakis, George Piaditis

LETTERS TO THE EDITOR

Landmarks in the history of adrenal surgery ........................................................................................................................................ 136
Marios Papadakis, Andreas Manios, Georgios Schoretsanitis, Constantinos Trompoukis

Does ambient light at night reduce total melatonin production? .......................................................................................................... 142
Christopher C.M. Kyba, Thomas Kantermann

Central precocious puberty due to hypothalamic hamartoma in neurofibromatosis type 1 ................................................................. 144
Emanuele Bartolini, Stefano Stagi, Perla Scalini, Andrea Bianchi, Antonio Ciccarone, Mario Mascalchi

Diversity in endocrinology practice: the case of Ramadan .................................................................................................................. 147
Ioannis Ilias, Luai Said Tayeh, Isidoros Pachoundakis

Depressive symptoms in obese patients study ...................................................................................................................................... 149
Bulent Canbaz
More news from IMPROVE-IT (IMProved Reduction of Outcomes: Vytorin Efficacy International Trial)

Niki Katsiki,1 Vasilios G. Athyros,1 Dimitris P. Mikhailidis2

1Second Propedeutic Department of Internal Medicine, Medical School, Aristotle University of Thessaloniki, Hippokration Hospital, Thessaloniki, Greece; 2Department of Clinical Biochemistry (Vascular Prevention Clinics), Royal Free Hospital Campus, University College Medical School, University College London, London, UK

The IMProved Reduction of Outcomes: Vytorin Efficacy International Trial (IMPROVE-IT) reported a significant reduction in first primary endpoint (PEP) post-acute coronary syndrome (ACS) in patients (n=18,144) on ezetimibe + simvastatin compared with placebo + simvastatin.1 The low-density lipoprotein cholesterol (LDL-C) levels in the combination group were significantly lower than in the monotherapy group (53.7 vs 69.5 mg/dl; 1.4 vs 1.8 mmol/l).

A recent analysis of IMPROVE-IT considered all PEP events [cardiovascular (CV) death, myocardial infarction (MI), stroke, unstable angina leading to hospitalization, coronary revascularization ≥30 days post-randomization].2 The duration of the trial was a median of 6 years (25th, 75th percentiles: 4.3 and 7.1 years). Often trials only consider the first vascular event.2 However, this may not represent the clinical situation, since patients with ACS may have recurrent events (except when the initial event is death). Assessing all events is an advantage in trials which last longer, as for IMPROVE-IT.

Not surprisingly, the all events analysis2 of IMPROVE-IT is more impressive than the initial first event only analysis.1 There were 9,545 total PEP events [5,314 (56%) first events + 4,231 (44%) subsequent events]. Thus, the first event analysis did not include >4000 events. Total PEP events were significantly reduced by 9% with ezetimibe/simvastatin vs placebo/simvastatin [incidence-rate ratio (RR): 0.91; 95% confidence interval (CI): 0.85 to 0.97; p=0.007]. There were an additional 251 fewer events in the combination therapy group after considering all events. This should be added to the 170 first events prevented making a total of 421 fewer events in the combination therapy group. This translates into 11 total PEP events prevented with ezetimibe plus simvastatin compared with simvastatin monotherapy for every 100 patients treated for 10 years.

In the all events analysis2 of IMPROVE-IT, the exploratory composite endpoint of CV death, MI or stroke was significantly reduced (RR: 0.88; 95% CI: 0.81 to 0.96; p=0.002). The reduction in total events was mainly driven by a decrease in non-fatal MI (RR: 0.87; 95% CI: 0.79 to 0.96; p=0.004) and non-fatal stroke (RR: 0.77; 95% CI: 0.65 to 0.93; p=0.005).

When the total number of PEP events were considered, 70.7% of the participants had no events, 16.6% had 1 event, 7.3% had 2 events and 5.4% had ≥3 events. The greatest number of events was 14 events in 2 patients; 13% of the 18,144 subjects had >1 PEP event.

Compared with patients with only 1 event, those with multiple events had more comorbidities at study
entry, including hypertension and diabetes mellitus (DM), and more had previously experienced MI, angina or revascularization.

The all events analysis\(^2\) of IMPROVE-IT also provides additional evidence for the LDL-C hypothesis (i.e. lower is better). The 1-month LDL-C levels were lowest in those without a subsequent PEP event and highest in those with >1 PEP event (mean 58.3 mg/dl for no event, 59.6 mg/dl for 1 event and 60.1 mg/dl for >1 event; \(p < 0.001\) for 3-way comparison). However, there was no difference when LDL-C levels in those with 1 PEP event were compared with those with >1 PEP event. An LDL-C of <70 mg/dl at 1 month was most common among subjects without a PEP event during the trial compared with subjects with 1 or >1 event (\(p < 0.001\) for 3-way comparison). However, there was no significant difference when comparing subjects with 1 PEP event with those who had >1 PEP event.

The all events analysis\(^2\) of IMPROVE-IT suggests that sustained long-term lipid lowering treatment that achieves low LDL-C levels is necessary to achieve a continuous decrease in events. However, this all events analysis\(^2\) also has limitations, as outlined by the authors. For example, after a first nonfatal event, many subjects discontinue their blinded study drug, which may influence subsequent events occurring off study drug. To address this limitation, an on-treatment analysis was performed\(^2\) which showed findings consistent with the intent to treat analysis.

The IMPROVE-IT results\(^1\) suggest a greater benefit for patients with DM in terms of event reduction. Furthermore, there was no increase in statin-associated new onset DM (NOD) in the combination therapy group despite achieving significantly lower LDL-C levels [http://www.tctmd.com/show.aspx?id=130400; abstract presented at the European Society of Cardiology meeting, London, August 2015]. There may be several reasons for this result.\(^3\) Because there are risk factors that increase the risk of NOD, it would be of interest to assess the incidence of NOD in patients with different numbers of these risk factors. The rationale is that there is some evidence of beneficial effects of ezetimibe on insulin resistance.\(^3\) As one predictor of statin-associated NOD is the duration of statin treatment, the duration of IMPROVE-IT (up to 7.1 years) may prove to be an advantage. There is also evidence that lowering LDL-C levels with statins helps preserve kidney function.\(^4\) This effect could extend to ezetimibe.\(^5,6\) However, this renal effect of statins or ezetimibe is only likely to be seen in those with some degree of chronic kidney disease. Again, the duration of IMPROVE-IT could prove useful because there is evidence that statins may not protect kidney function in long-term studies.\(^7\)

The cost effectiveness of ezetimibe will increase when this drug becomes a generic product in several countries within the next few years. This drug remains the only add-on to a statin with convincing event-based evidence.\(^8\)

It is surprising that with this level of evidence the Endocrinologic and Metabolic Drugs advisory committee of the food and drug administration did not accept the recommendation of ezetimibe use as add-on to statin therapy in coronary heart disease patients [http://www.medscape.com/viewarticle/855958?nlid =936032566&src=wnl_edit_medp_card&uac=182515FR&spon=2&impID=921006&fafal=1]. In contrast, the European Society of Cardiology guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-Segment elevation recommend ezetimibe as the only add-on to statins.\(^9\)

A survey of a large health care system (n=219,625 patients with ACS) concluded that 31.6% could qualify for ezetimibe if used outside of the strict IMPROVE-IT trial inclusions.\(^10\) It follows that defining the role of ezetimibe in patients with ACS already taking high-intensity statins or those with statin intolerance is important. In this context, statin intolerance remains a clinically relevant problem.\(^11\)

Undoubtedly there will be further sub-analyses of the IMPROVE-IT trial. Obviously, there is a need to consider the effect of additional events on quality of life and health expenditure. An economic analysis of IMPROVE-IT has been planned.

REFERENCES

Expert consensus on the rational clinical use of proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors

Apostolos Achimastos, 1 Theodoros Alexandrides, 2 Dimitrios Alexopoulos, 3 Vasilios Athyros, 4 Alexandra Bargiota, 5 Eleni Bilianou, 6 Christina Chrysochoou, 7 Evridiki Drogari, 8 Moses Elisaf*, 9 Emanouel Ganotakis, 10 Ioannis Goudevenos, 11 Ioannis Ioannidis, 12 Genovefa Kolovou, 13 Vasilios Kotsis, 14 Ioannis Lekakis, 15 Evangelos Liberopoulos, 16 Andreas Melidonis, 17 Vasilios Nikolou, 18 George Ntaios, 19 Nikolaos Papanas, 20 Stavros Pappas, 21 Christos Pitsavos, 22 Loukianos Rallidis, 23 Dimitrios Richter, 24 Ioannis Skoumas, 25 Nicolaos Tentolouris, 26 Dimitrios Tousoulis, 27 Alexandros Tselepis, 28 Konstantinos Tsioufis, 29 Dimitrios Tziakas, 30 Konstantinos Tzimalos, 31 Panagiotis Vardas, 32 Charalabos Vlachopoulos, 33 Dimitrios Vlahakos 34

1 Professor of Internal Medicine, Medical School, National and Kapodistrian University of Athens, 2 Professor of Internal Medicine-Endocrinology, Medical School, University of Patras, 3 Professor of Cardiology, Medical School, University of Patras, 4 Associate Professor of Internal Medicine, Past President of the Hellenic Atherosclerosis Society Aristotle University of Thessaloniki, 5 Assistant Professor of Internal Medicine-Endocrinology, Medical School, University of Thessaly, 6 Consultant-Cardiology, Head of Lipid Outpatient Clinic, “Tzanieio” General Hospital of Piraues, 7 Consultant Cardiology, 1st University Cardiology Clinic, “Hippokratio” General Hospital of Athens, 8 Assistant Professor of Metabolic Pediatrics, Medical School, National and Kapodistrian University of Athens, 9 Professor of Internal Medicine, Medical School, Past President of the Hellenic Atherosclerosis Society, University of Ioannina, 10 Professor of Internal Medicine, Medical School, Past President of the Hellenic Atherosclerosis Society, University of Crete, 11 Professor of Cardiology, Medical School, University of Ioannina, 12 Consultant-Internal Medicine, Head of the Diabetics and Obesity Outpatient Clinics, General Hospital of Nea Ionia “Konstantopouleia-Patission”, 13 Consultant-Cardiology, Head of LDL Apheresis Unit and Lipid Outpatient Clinics, Past President of the Hellenic Atherosclerosis Society, President of the Hellenic College of Treatment of Atherosclerosis, Onassis Cardiac Surgery Center, 14 Assistant Professor of Internal Medicine, Aristotle University of Thessaloniki, 15 Professor of Cardiology, Medical School, National and Kapodistrian University of Athens, 16 Assistant Professor of Internal Medicine, Medical School, University of Ioannina, 17 Consultant-Internal Medicine, Coordinator-Director of 1st Internal Medicine Clinic, “Tzanieio” General Hospital of Piraues, 18 Consultant-Cardiology, “Korgialenio Benakio” “HRC” Hospital, Athens, 19 Assistant Professor of Internal Medicine, Medical School, University of Thessaly, 20 Associate Professor of Internal Medicine, Medical School, Democritus University of Thrace, 21 Internist-Diabetologist, President of the Institute for the Study, Research and Training on Diabetes Mellitus and Metabolic Diseases, Athens, 22 Emeritus Professor of Cardiology, Medical School, National and Kapodistrian University of Athens, 23 Associate Professor of Cardiology, Medical School, University of Athens, 24 Director of Cardiology Department, Athens Euroclinic, President of the Hellenic Society of Lipidology and Atherosclerosis, Athens, 25 Consultant-Cardiology, Head of the Lipid Unit of the 1st

Address for correspondence:
Moses Elisaf, Professor of Internal Medicine, Medical School, University of Ioannina, 451 10 Ioannina, Greece; Tel.: +30 2651-007 509, Fax: +30 2651-007 016, E-mail: melisaf54@gmail.com
Received: 04-12-2015, Accepted: 09-12-2015
**ABSTRACT**

Two proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, evolocumab and alirocumab, have recently been approved by both the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of hypercholesterolemia. These fully human monoclonal antibodies selectively block PCSK9, thus permitting the low-density lipoprotein (LDL) receptor to effectively recycle to the surface of liver cells. The administration of these antibodies leads to robust LDL cholesterol (LDL-C) lowering by 50-60% on top of maximum hypolipidemic treatment. At least 4 randomized, placebo-controlled studies are under way and will address the question of whether the administration of these PCSK9 inhibitors is associated with a significant reduction of cardiovascular events. Because of the high cost associated with the use of these medications it is very important to consider which patients may gain the most benefit, at least until the results of outcome studies are available. In this Consensus paper, 34 clinicians/scientists define 3 groups of patients that should be currently considered as candidates for the use of these novel drugs. These include: 1a. Adults with established cardiovascular disease and LDL-C >100 mg/dL while on lifestyle modifications and maximally tolerated hypolipidemic treatment, i.e. high-intensity statin + ezetimibe, 1b. Adults with diabetes and established cardiovascular disease or chronic kidney disease or target organ damage and LDL-C ≥100 mg/dL while on lifestyle modifications and maximally tolerated hypolipidemic treatment, i.e. high-intensity statin + ezetimibe, 2. Adults with familial hypercholesterolemia (FH) without established cardiovascular disease and LDL-C ≥130 mg/dL while on lifestyle modifications and maximally tolerated hypolipidemic treatment, i.e. high-intensity statin + ezetimibe (evolocumab is also indicated in children above 12 years with homozygous FH), and 3. Adults at high or very high cardiovascular risk who are statin intolerant and have an LDL-C ≥100 and ≥130 mg/dL, respectively, while on any tolerated hypolipidemic treatment.

**Key words:** Cardiovascular disease, Diabetes, Ezetimibe, Familial hypercholesterolemia, High-risk, LDL cholesterol, PCSK9, Statin intolerance, Statins

**INTRODUCTION**

Proprotein convertase subtilisin/kexin type 9 (PCSK9) plays an important role in lipoprotein metabolism because it binds and accelerates the cellular degradation of low-density lipoprotein (LDL) receptors, thus preventing their recycling to the hepatocyte surface. This effect results in the increase of plasma LDL cholesterol (LDL-C) levels.1-7

The administration of fully human monoclonal antibodies that bind plasma PCSK9 of patients treated with statins (with or without ezetimibe) results in an additional reduction of LDL-C by 50-60% and the achievement of lipid-lowering therapy goals in the vast majority of high-risk patients.7-19 Clinical and experimental data have shown that statins, as opposed to their ability to reduce LDL-C levels, increase
PCSK9 levels. This increase is due to the activation of the Sterol Regulatory Element-Binding-Protein-2 (SREBP-2), a transcription factor that induces gene expression and therefore increases the levels of LDL receptors as well as of PCSK9. These findings reinforce the clinical trial data showing that inhibition of PCSK9 with monoclonal antibodies enhances the lipid-lowering effect of statins. Moreover, it has been shown that PCSK9 inhibitors decrease levels of lipoprotein (a) [Lp(a)] by approximately 25%, triglycerides by 9%, non-high density lipoprotein (HDL) cholesterol by 52% and apolipoprotein B by 43% as well as increase HDL cholesterol (HDL-C) levels by 9% and apolipoprotein AI by 5%.

Three large meta-analyses of PCSK9 inhibitors, evolocumab and alirocumab (meta-analysis of 24 randomized studies by Navarese et al, 25 randomized studies by Zhang et al and 17 randomized studies by Lipinski et al), confirmed the efficacy and safety of both drugs, without differences regarding serious adverse events among patients treated with evolocumab or alirocumab and placebo. Of note, injection-site reactions and neurocognitive adverse events were more frequent in patients on PCSK9 inhibitors compared with placebo. The possible association of PCSK9 inhibitors with neurocognitive adverse events is under scrutiny in ongoing studies.

It is of particular interest that two studies recently published (ODYSSEY LONG TERM & OSLER studies) showed that administration of both drugs, apart from the well tolerated reduction in LDL-C, resulted in a significant (50%) reduction in cardiovascular (CV) events (treatment duration 1 year, starting LDL-C levels of ~120 mg/dL). However, these studies were not designed to evaluate the effect of the drugs on CV events, while the number of CV events recorded during treatment was low and the time of prospective follow-up of patients was limited. Ongoing studies will answer the question whether the reduction of LDL-C through PCSK9 inhibition leads to a proportional reduction of CV events in cases of long-term administration, without adverse events.

Regulatory authorities in the USA and Europe [Food and Drug Administration (FDA) and European Medicines Agency (EMA)] have approved the administration of these drugs to adult patients with primary hypercholesterolemia (heterozygous familial and non-familial) or mixed dyslipidemia, as adjunct treatment to diet:

- In combination with a statin or statin with other lipid lowering therapies in patients unable to reach LDL-C goals with the maximum tolerated dose of a statin, or,
- Alone or in combination with other lipid-lowering therapies in patients who are statin-intolerant or for whom a statin is contraindicated.

Evolocumab has received an additional indication in adolescents over 12 years old with homozygous familial hypercholesterolemia (FH) in combination with other lipid-lowering treatments.

The drugs are administered as subcutaneous injections every 2 weeks (evolocumab 140 mg and alirocumab 75 or 150 mg) or 4 weeks (evolocumab 420 mg).

Patients who could benefit from treatment with PCSK9 inhibitors

Until the announcement-publication of the prospective randomized clinical trials that will confirm the effect of these drugs on CV morbidity and mortality, it is proposed that their prescription be limited to specific patient groups at very high risk who are expected to benefit from the treatment.

The following guidelines are consistent with the recent recommendations of the US National Lipid Association (NLA) and are summarized in Table 1. It is to be noted that patient adherence with already administered lipid-lowering therapy should always be examined first. The patient groups that are expected to benefit from treatment are: (1) patients with FH, (2) patients with established vascular disease and very high-risk diabetic patients who do not achieve the hypolipidemic treatment goals with the maximum available lipid-lowering therapy (high doses of effective statins + ezetimibe), and (3) patients intolerant to statins. Patients with FH are at high CV risk because of very high levels of LDL-C. These patients often do not achieve the goals of hypolipidemic treatment in daily clinical practice. It must be underlined that a percentage of patients display intolerance to statins, i.e. myalgia with or without an increase in muscle
Table 1. Profiles of eligible patients for administration of monoclonal antibodies against PCSK9 until the completion of large randomized clinical trials with cardiovascular outcomes

<table>
<thead>
<tr>
<th>Group of high- to very high-risk individuals</th>
<th>Ultimate treatment goal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a. Adult patients with established atherosclerotic CV disease (coronary, carotid or peripheral vessels) and LDL-C ≥100 mg/dL</td>
<td>Under appropriate health-diet and pharmaceutical treatment with the maximum tolerated dose of effective statin (atorvastatin 40/80 mg or rosvastatin 20/40 mg) + ezetimibe 10 mg</td>
</tr>
<tr>
<td>1b. Diabetic patients with known CV disease or chronic kidney disease (estimated glomerular filtration rate ≤60 mL/min/1.73 m² and/or albuminuria for at least 3 months) or other target organ damage and LDL-C ≥100 mg/dL</td>
<td>Under treatment with the maximum tolerated dose of effective statin (atorvastatin 40/80 mg or rosvastatin 20/40 mg) + ezetimibe 10 mg</td>
</tr>
<tr>
<td>2. Adult patients FH without known atherosclerotic cardiovascular disease and LDL-C ≥130 mg/dL*</td>
<td>Under any tolerated lipid-lowering treatment</td>
</tr>
<tr>
<td>3. High- or very high-risk patients (HELLENIC SCORE &gt;5% or &gt;10%, respectively) who are intolerant to statins and have LDL-C ≥130 or ≥100 mg/dL, respectively</td>
<td></td>
</tr>
</tbody>
</table>

* Evolocumab has received additional indication in adolescents over 12 years old with homozygous FH in combination with other lipid-lowering treatments.

Table 2. Dutch Lipid Clinic Network diagnostic criteria for Familial Hypercholesterolemia

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>First degree relative with:</td>
<td></td>
</tr>
<tr>
<td>• Known premature coronary or vascular disease (men aged &lt;55 years, women aged &lt;60 years)</td>
<td>1</td>
</tr>
<tr>
<td>• LDL-C above the 95th percentile for age and gender</td>
<td>1</td>
</tr>
<tr>
<td>• Tendinous xanthomata and/or corneal arcus</td>
<td>2</td>
</tr>
<tr>
<td>First degree relative &lt;18 years with LDL-C above the 95th percentile for age and gender</td>
<td>2</td>
</tr>
<tr>
<td>Patients with premature coronary artery disease (men aged &lt;55 years, women aged &lt;60 years)</td>
<td>2</td>
</tr>
<tr>
<td>Patients with premature peripheral arterial disease or ischemic stroke (men aged &lt;55 years, women aged &lt;60 years)</td>
<td>1</td>
</tr>
<tr>
<td>Tendinous xanthomata</td>
<td>6</td>
</tr>
<tr>
<td>Corneal arcus prior to age 45 years</td>
<td>4</td>
</tr>
<tr>
<td>LDL-C, mg/dL (mmol/L)</td>
<td></td>
</tr>
<tr>
<td>• ≥330 (≥8.5)</td>
<td>8</td>
</tr>
<tr>
<td>• 250-329 (6.5-8.4)</td>
<td>5</td>
</tr>
<tr>
<td>• 190-249 (5.0-6.4)</td>
<td>3</td>
</tr>
<tr>
<td>• 155-189 (4.0-4.9)</td>
<td>1</td>
</tr>
<tr>
<td>Functional mutation in the LDL receptor, apolipoprotein B or PCSK9 gene</td>
<td>8</td>
</tr>
<tr>
<td>Diagnosis</td>
<td></td>
</tr>
<tr>
<td>&gt;8</td>
<td>Definite FH</td>
</tr>
<tr>
<td>6-8</td>
<td>Probable FH</td>
</tr>
<tr>
<td>3-5</td>
<td>Possible FH</td>
</tr>
</tbody>
</table>

Diagnosis of FH

The diagnosis of heterozygous FH is set clinically using the Dutch criteria (Table 2 and FH score in enzymes, which makes it difficult to continue treatment with statins or to administer high doses of these drugs.21-25
App Store (https://appsto.re/gr/wF4Q7.i), FH score in Google Store (https://play.google.com/store/apps/details?id=com.ajjumax.helleniccalculator) and Download in desktop (http://web.alphabit.gr/FHCalculator/index.html) and when the patient has a score ≥6 (probable or definite FH).26-38

The Hellenic Atherosclerosis Society has already initiated a nationwide registry of patients with FH (HELLAS FH Registry - Hellenic Registry of Patients with FH). The inclusion of any patient in this registry also confirms its diagnosis.

**Diagnosis of statin intolerance**

The diagnosis of statin intolerance is set in patients who: (a) display significant increases in creatine kinase (CK) >5 times of the upper limit reference values, and/or, (b) irrespective of any increase in CK, display muscle symptoms which may be attributed to statins (pain, fatigue, weakness, cramps) and after the exclusion of any other factors which could cause similar symptoms (Table 3) and/or any possible interactions of the co-administered drugs have been excluded (Table 4).

In order to confirm possible statin intolerance, a sequential administration of at least 2 different statins starting at low doses followed by a careful dosage up-titration over a few weeks is required (Table 5). The improvement of symptoms following statin treatment discontinuation and their reappearance with the re-administration of the same or a different statin reinforce the diagnosis of statin intolerance. The therapeutic options in patients intolerant to statins before administration of the PCSK9 inhibitors are shown in Table 6.21-25

Table 3. Causes to be excluded in patients with muscular pains and/or CK increase before these findings are attributed to statins.

<table>
<thead>
<tr>
<th>Cause</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Exercise-Muscle strain</td>
</tr>
<tr>
<td>2. Intramuscular injections</td>
</tr>
<tr>
<td>3. Drugs (cocaine, heroin, amphetamines) - Alcohol</td>
</tr>
<tr>
<td>4. Hypothyroidism</td>
</tr>
<tr>
<td>5. Infections</td>
</tr>
<tr>
<td>6. Electrolyte disorders (e.g. hypokalemia)</td>
</tr>
<tr>
<td>7. Metabolic myopathies</td>
</tr>
<tr>
<td>8. Inflammatory and autoimmune myositis</td>
</tr>
</tbody>
</table>

Table 4. Drug interactions of statins

<table>
<thead>
<tr>
<th>Drug interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Fibrates (mainly gemfibrozil - not fenofibrate)</td>
</tr>
<tr>
<td>2. Coumarin anticoagulants</td>
</tr>
<tr>
<td>3. Cyclosporine</td>
</tr>
<tr>
<td>4. Erythromycin and other macrolides (clarithromycin)</td>
</tr>
<tr>
<td>5. Itraconazole and other antifungal medicines</td>
</tr>
<tr>
<td>6. Antidepressants (nefazodone)</td>
</tr>
<tr>
<td>7. Protease inhibitors</td>
</tr>
<tr>
<td>8. Dihydropyridines, as well as diltiazem/verapamil (mainly with simvastatin)</td>
</tr>
<tr>
<td>9. Amiodarone (mainly with simvastatin/lovastatin)</td>
</tr>
<tr>
<td>10. Grapefruit juice</td>
</tr>
<tr>
<td>11. Drugs that induce the activity of CYP3A4 (phenytoin, rifampicin)</td>
</tr>
</tbody>
</table>

Table 5. Statins marketed in Greece (approved dose range)

<table>
<thead>
<tr>
<th>Statin</th>
<th>Dose range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atorvastatin</td>
<td>10–80 mg</td>
</tr>
<tr>
<td>Lovastatin</td>
<td>20–80 mg</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>1–4 mg</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>10–40 mg</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>5–40 mg</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>10–40 mg</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>20–80 mg</td>
</tr>
</tbody>
</table>

Table 6. Therapeutic options in patients intolerant to statins prior to the administration of PCSK9 inhibitors.

<table>
<thead>
<tr>
<th>Option</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Aggressive health-diet changes.</td>
</tr>
<tr>
<td>2. Administration of ezetimibe (10 mg/day).</td>
</tr>
<tr>
<td>3. Administration of a combination of ezetimibe (10 mg/day) with colesevalam (3.75 g/day). The expected reduction in LDL-C is 30%. Alternatively, a combination of ezetimibe with fenofibrate may be administered.</td>
</tr>
<tr>
<td>4. Potential careful administration of pravastatin 20 mg/day or fluvastatin 40 mg/day.</td>
</tr>
<tr>
<td>5. Administration of rosuvastatin 5 mg or atorvastatin 10 mg every other day or twice per week or once per week, in combination with daily ezetimibe.</td>
</tr>
<tr>
<td>6. Measurement of vitamin 25(OH)D; levels and supplementation in cases of reduced levels, although the evidence is limited.</td>
</tr>
<tr>
<td>7. Dealing with the factors listed in Table 3.</td>
</tr>
</tbody>
</table>

**FINANCIAL AND COMPETING INTEREST DISCLOSURE**

This consensus was written independently and
was not financed by the pharmaceutical industry. Some authors have given lectures and participated in congresses and advisory boards of various pharmaceutical companies; however, they have no conflict of interest in terms of employment, stock ownership or options, grants, or patents received or pending, or royalties. No writing assistance was utilized in the production of this consensus.

ACKNOWLEDGEMENT

This consensus was published in Greek in the Hellenic Journal of Atherosclerosis (Hellenic J Atheroscler 2015, 6(Suppl): 3-8). After agreement of the two Editors-in-Chief it is published in English in an extended form in Hormones.

REFERENCES


Neoadjuvant therapy for advanced pancreatic neuroendocrine tumors: an emerging treatment modality?

Iraklis Perysinakis,1 Chrysanthi Aggeli,1 Gregory Kaltsas,2 George N. Zografos1

1Third Department of Surgery, General Hospital “G. Gennimatas”; 2Department of Pathophysiology, National University of Athens; Athens, Greece

ABSTRACT

OBJECTIVE: Complete surgical resection is the only potentially curative treatment of localized pancreatic neuroendocrine tumors. Unfortunately, a significant proportion of these patients present with unresectable locally advanced tumors or massive metastatic disease. Recently, a new therapeutic approach for this subset of patients has emerged consisting of neoadjuvant therapy followed by surgical exploration in responders. DESIGN: We searched MEDLINE for the purpose of identifying reports regarding neoadjuvant treatment modalities for advanced pancreatic neuroendocrine tumors. RESULTS: We identified 12 studies, the vast majority of which were either case reports or small case series. Treatment options included chemotherapy, radiotherapy, peptide receptor radionuclide therapy, biological agents or various combinations of them. CONCLUSIONS: Increasing evidence supports the application of neoadjuvant protocols in advanced pancreatic neuroendocrine tumors aiming at tumor downsizing, thus rendering curative resection feasible. Given that prospective and controlled randomized clinical trials from high-volume institutions are not feasible, expert panel consensus is needed to define the optimal treatment algorithm.

Key words: Locally advanced-liver metastasis, Neoadjuvant therapy, Pancreatic neuroendocrine tumor

INTRODUCTION

Pancreatic neuroendocrine tumors (pNETs) are uncommon neoplasms that represent 1-2% of all pancreatic neoplasms.1 The World Health Organization (WHO) classification of 2010 adopted the European Neuroendocrine Tumor Society (ENETS) grading system, categorizing NETs as NET G1 (low grade), NET G2 (intermediate grade) and poorly differentiated neuroendocrine carcinomas (NECs).2,3 A substantial percentage of pNETs (65-80%) is associated with malignant behavior and recurrence following resection. However, in many cases disease progression may be very slow resulting in prolonged survival.1 pNETs are currently staged according to the American Joint Committee on Cancer (AJCC) classification of 2010 (7th edition).4

Complete surgical resection (R0 excision) is the only potentially curative treatment of localized pNETs.
While an aggressive surgical approach has been advocated, surgical debulking or planned R2 resection have not received wide support.

Unfortunately, a significant proportion of patients with pNETs present with unresectable locally advanced tumors or massive metastatic disease, which render surgical treatment unfeasible.

Recently, a new therapeutic approach for this subset of patients with inoperable pNETs has emerged consisting of induction therapy in the neoadjuvant setting followed by surgical exploration in responders. Neoadjuvant chemotherapy has been extensively used for locally advanced adenocarcinomas with remarkable clinical results over the last few decades. With regard to pNETs, this approach is occasionally considered and suggested by several authors, mainly through case reports or small case series.

The purpose of this review is to update current knowledge regarding neoadjuvant treatment of locally advanced pNETs. We carried out a comprehensive search of the literature, using PubMed (http://www.ncbi.nlm.nih.gov/pubmed). The following keywords were used in the search: pancreatic neuroendocrine tumors, locally advanced, neoadjuvant therapy, preoperative chemotherapy, preoperative radiotherapy. No language restrictions were applied. The PubMed search was extended up to April 2015 to retrieve the latest additional publications. Moreover, the bibliographies of reviewed articles were scrutinized to obtain any other references that eluded the primary search. Original research articles [randomized controlled trials (RCTs), prospective and retrospective studies], meta-analyses, reviews, editorials, commentaries, case reports, case series and letters were included.

DEFINITIONS OF LOCALLY ADVANCED PANCREATIC TUMORS

Locally advanced pancreatic cancers are defined as tumors adherent to or invading adjacent structures, including celiac and superior mesenteric vasculature (T4 or stage III disease). Recently, two distinct subgroups of such tumors have been identified: borderline resectable and locally advanced unresectable pancreatic tumors.

According to the consensus-based guidelines from the National Comprehensive Cancer Network (NCCN), criteria for unresectability are as follows:

- For pancreatic head and body tumors: greater than 180 degrees superior mesenteric artery (SMA) encasement or any celiac artery abutment, unreconstructable superior mesenteric vein (SMV)/portal vein (PV) occlusion, aortic invasion or encasement.
- For pancreatic tail tumors: greater than 180 degrees SMA encasement or any celiac artery abutment.
- For all sites: distant metastases, metastases to lymph nodes beyond the field of resection.

The definition of borderline resectable tumors is variable, mainly due to differences between centers in feasibility of SMV reconstruction. However, the most commonly cited criteria are those recommended by a consensus statement of the American Hepato-Pancreato-Biliary Association/Society of Surgical Oncology/Society for Surgery of the Alimentary Tract, which have also been included in the guidelines of the NCCN. According to this definition, borderline resectable pancreatic tumors present the following characteristics:

- No distant metastases.
- Venous involvement of the SMV/PV demonstrating tumor abutment with or without impingement and narrowing of the lumen, encasement of the SMV/PV but without encasement of the nearby arteries, or short segment venous occlusion resulting from either tumor thrombus or encasement, but with suitable vessel proximal and distal to the area of vessel involvement, allowing for safe resection and reconstruction.
- Gastroduodenal artery encasement up to the hepatic artery with either short segment encasement or direct tumor abutment of the hepatic artery, without extension to the celiac axis.
- Tumor abutment of the SMA not to exceed >180 degrees of the circumference of the vessel wall.

Concerning neuroendocrine tumors, the ENETS has set up a tumor-node-metastasis (TNM) staging system as well as a grading system (G1, G2, and G3) (Table 1).
Neoadjuvant therapy for advanced pancreatic neuroendocrine tumors

Progression despite therapy who are not expected to benefit from surgery.

Two meta-analyses including series of patients with pancreatic adenocarcinoma have concluded that approximately one third of patients with locally advanced, unresectable or borderline resectable tumors can be resected after neoadjuvant therapy, with survival rates comparable to those of patients with initially resectable tumors.14,15 Therapeutic options include chemotherapy, chemoradiotherapy or a combined approach. Radiotherapy alone has been tried to a lesser extent. However, the optimal regimen in this setting is not to date established.9

The role of neoadjuvant therapy in the treatment of advanced pNETs

In regard to neuroendocrine pancreatic tumors, either synchronous or metachronous resection of the primary and metastatic tumors is recommended to be performed whenever possible.12,16,17 Even in the setting of locally advanced tumor and/or metastatic disease, surgery may be the treatment of choice aiming at tumor reduction and palliation of mass effect or hormone-related symptoms.18 Surgical excision should be performed only if more than 90% of the tumor mass can be resected.19 On the other hand, it has been suggested that palliative debulking surgery has no significant effect on survival as compared to palliation without surgery.20

Regarding inoperable pNETs, the current guidelines suggest observation for patients with pNETs G1/G2 who are asymptomatic, with low tumor burden and stable disease. In the case of symptomatic patients with large tumor volume or progressive disease, first-line therapy recommendations include biological agents (sunitinib, everolimus), chemotherapy, arterial embolization, chemoembolization, ablative therapy, cytoreductive surgery, supportive medical care and somatostatin analogs. Patients with inoperable pancreatic NECs should be started on cisplatin- or etoposide-based chemotherapy or offered the chance to participate in clinical trials.18

During the last decade, several institutions have reported response rates of 39% to 71% with nonsurgical treatments in patients with advanced pNETs,21 although the majority of these published studies are

Table 1. Grading proposal for foregut neuroendocrine tumors from ENETS5

<table>
<thead>
<tr>
<th>Grade</th>
<th>Mitotic count (10 HPF)a</th>
<th>Ki-67 index (%)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>&lt;2</td>
<td>≤2</td>
</tr>
<tr>
<td>G2</td>
<td>2-20</td>
<td>3-20</td>
</tr>
<tr>
<td>G3</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
</tbody>
</table>

*a10 HPF: high power field=2 mm², at least 40 fields (at 40 × magnification) evaluated in areas of highest mitotic density; bMIB1 antibody; % of 2,000 tumor cells in areas of highest nuclear labeling.

In addition, the AJCC has proposed a TNM staging classification significantly different to the ENETS staging system.4 From the surgical point of view, the AJCC staging system incorporates in T4 tumor assessment the importance of anatomical correlation of the tumor with the adjacent vascular structures, which is the cornerstone of resectability in pancreatic neuroendocrine tumors. Of note, the ENETS tumor staging system, which equates tumor infiltration of viscera with major vascular involvement, is not compatible with current clinical practice. It should be stressed that preoperative imaging studies showing possible vascular involvement as well as definite detection of this intraoperatively are usually considered contraindications to surgery.

Aggressive surgery for T4 tumors including superior mesenteric vein reconstruction can be contemplated, but the surgical risk-benefit ratio should be carefully weighed.12,13

Oncologic perspectives

Nowadays, the use of neoadjuvant therapy is an established treatment in patients with pancreatic ductal adenocarcinoma (PDAC). PDAC carries the worst prognosis of all malignancies of the alimentary tract. Despite recent advances in imaging studies, only 10 to 20% of patients have resectable disease at the time of presentation. Of the remaining patients, 30 to 40% present with locally advanced tumors. Median survival for these patients is 8-12 months.9

Due to their poor prognosis, such patients are candidates for neoadjuvant therapy with the aim of tumor downsizing (or even disease downstaging) and subsequent resection. Moreover, neoadjuvant therapy is better tolerated by patients and allows for the identification of those patients with rapid disease progression despite therapy who are not expected to benefit from surgery.

Two meta-analyses including series of patients with pancreatic adenocarcinoma have concluded that approximately one third of patients with locally advanced, unresectable or borderline resectable tumors can be resected after neoadjuvant therapy, with survival rates comparable to those of patients with initially resectable tumors.14,15 Therapeutic options include chemotherapy, chemoradiotherapy or a combined approach. Radiotherapy alone has been tried to a lesser extent. However, the optimal regimen in this setting is not to date established.9

The role of neoadjuvant therapy in the treatment of advanced pNETs

In regard to neuroendocrine pancreatic tumors, either synchronous or metachronous resection of the primary and metastatic tumors is recommended to be performed whenever possible.12,16,17

Even in the setting of locally advanced tumor and/or metastatic disease, surgery may be the treatment of choice aiming at tumor reduction and palliation of mass effect or hormone-related symptoms.18 Surgical excision should be performed only if more than 90% of the tumor mass can be resected.19 On the other hand, it has been suggested that palliative debulking surgery has no significant effect on survival as compared to palliation without surgery.20

Regarding inoperable pNETs, the current guidelines suggest observation for patients with pNETs G1/G2 who are asymptomatic, with low tumor burden and stable disease. In the case of symptomatic patients with large tumor volume or progressive disease, first-line therapy recommendations include biological agents (sunitinib, everolimus), chemotherapy, arterial embolization, chemoembolization, ablative therapy, cytoreductive surgery, supportive medical care and somatostatin analogs. Patients with inoperable pancreatic NECs should be started on cisplatin- or etoposide-based chemotherapy or offered the chance to participate in clinical trials.18

During the last decade, several institutions have reported response rates of 39% to 71% with nonsurgical treatments in patients with advanced pNETs,21 although the majority of these published studies are
single-arm, non-randomized ones with a small number of patients and therefore do not report clinically meaningful outcomes. Nevertheless, a subgroup of these patients with initially inoperable tumors turned out to be resectable as a result of significant downsizing caused by systemic therapy. This fact along with the substantial recurrence rates reported after surgical approaches point to the benefit of neoadjuvant concepts in patients with advanced pNETs.

Helical CT with 3-dimensional reconstruction and magnetic resonance visceral angiogram are used to assess resectability of pancreatic tumors following neoadjuvant chemotherapy. A recent large series of patients who presented with inoperable or borderline resectable pancreatic adenocarcinoma and received neoadjuvant folfirinox with or without chemo-radiotherapy demonstrated impressive improvements.

The well documented result of the study shows that traditional imaging criteria for resectability following neo-adjuvant therapy were not accurate and the authors suggested serial intraoperative biopsies around the involved vascular structures before attempting resection. In the field of neuroendocrine pancreatic tumors, Norton et al demonstrated that radiological abutment or even possible vascular involvement is not frequently synonymous with vascular involvement at surgery.

The potential role of induction therapy in advanced pNETs has been assessed only in a limited number of studies (Table 2), of which the vast majority are case reports. In other retrospective studies which have included larger series of patients, the therapeutic regimen was not given initially in the neoadjuvant setting. Surgical exploration following therapy was undertaken in only a few selected patients that presented the best response. Therapeutic options that have been studied include chemotherapy, peptide receptor radionuclide therapy (PRRT), biological agents and radiotherapy.

Much controversy exists over the appropriate term that should be used to describe the effect of neoadjuvant treatment on disease. Although most authors use terms such as: tumor downsizing, reduction, shrinkage or partial response. There have also been reports employing the term: disease downstaging. Such publications should be judged cautiously because in some of them the term has proven to have been used inappropriately. Moreover, the primary goal of neoadjuvant therapy should not be disease downstaging, but tumor downsizing in order to render it operable.

**CHEMOTHERAPY**

Until lately, neuroendocrine tumors have not usually been considered the ideal target for traditional DNA-damaging cytotoxic agents. Sorbye et al first reported in 2007 a patient with pancreatic NEC with liver metastases who responded partially to induction chemotherapy with etoposide plus cisplatin and underwent complete resection. Interestingly, no primary tumor was found in the pancreactectomy specimen. The patient received adjuvant therapy and was alive and free of disease 5 years after the operation.

Lessing et al in 2001 described 3 patients with extrapancreatic NECs who received neoadjuvant therapy with etoposide plus cisplatin. The first patient presented partial response and underwent complete resection. Eighteen months postoperatively the patient is alive and free of disease. The second patient also underwent complete resection after presenting partial response, but died 5 months postoperatively with local recurrence. Complete response was noted in the third patient and no mass was found in surgical exploration. However, the patient had local recurrence one year after the operation.

Sato et al reported a patient with pNET and multiple liver metastases that were treated with S-1, an oral fluorinated pyrimidine which contains tegafur, a prodrug of 5-FU, 5-chloro-2,4-dihydroxypyridine and potassium oxonate. The primary tumor presented partial response, while liver metastases presented complete response, allowing complete resection. The patient was alive and free of disease 6 months after the operation. It must be stressed that this is the only report of disease downstaging after neoadjuvant treatment, since, according to the authors, there was complete disappearance of liver metastases.

A very recent retrospective study by Dumont et al included 42 patients with locally advanced pNETs G1/G2 and segmental portal hypertension who were treated with different chemotherapeutic regimens containing 5-FU, streptozocin, doxorubicin, cisplatin,
<table>
<thead>
<tr>
<th>Author, year</th>
<th>No of pts(^a)</th>
<th>Disease characteristics</th>
<th>Induction therapy</th>
<th>Comments</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorbye et al, 2007(^{25})</td>
<td>1</td>
<td>Pancreatic NEC, liver metastasis</td>
<td>Etoposide plus cisplatin</td>
<td>Partial response. R0 resection, no primary tumor found in pancreatectomy specimen</td>
<td>Adjuvant therapy 5 years - free of disease</td>
</tr>
<tr>
<td>Kwekkeboom et al, 2008(^{30})</td>
<td>4/310</td>
<td>Non-functioning pNETs</td>
<td>PRRT ((^{177})Lu-octreotate)</td>
<td>Partial response. R0 resection. One died postoperatively from complications</td>
<td>nr</td>
</tr>
<tr>
<td>Kaemmerer et al, 2009(^{34})</td>
<td>1</td>
<td>Pancreatic NEC</td>
<td>PRRT ((^{90})Y-DOTA-TATE)</td>
<td>Partial response. R0 resection</td>
<td>18 months - free of disease</td>
</tr>
<tr>
<td>Stoeltzing et al, 2010(^{31})</td>
<td>1</td>
<td>Resected pNET, bilobular liver metastases</td>
<td>PRRT ((^{90})Y-DOTA-TOC)</td>
<td>Partial response. R0 resection of liver metastases</td>
<td>12 months - free of disease</td>
</tr>
<tr>
<td>Sato et al, 2010(^{27})</td>
<td>1</td>
<td>pNET, multiple liver metastases</td>
<td>S-1</td>
<td>Partial response (primary tumor)- complete response (liver metastases). R0 resection</td>
<td>6 months - free of disease</td>
</tr>
<tr>
<td>Sowa-Staszczak et al, 2011(^{33})</td>
<td>2/6</td>
<td>pNETs, 1 with liver metastases</td>
<td>PRRT ((^{90})Y-DOTA-TATE)</td>
<td>Partial response (primary tumor). R2 resection Partial response (primary tumor) - complete response (liver metastases). R0 resection</td>
<td>nr</td>
</tr>
<tr>
<td>Lessing et al, 2011(^{26})</td>
<td>3</td>
<td>NECs (2 duodenum, 1 rectosigmoid)</td>
<td>Etoposide, cisplatin</td>
<td>Partial response. R0 resection. R0 resection Complete response. No tumor at exploration</td>
<td>18 months – free of disease 5 months – recurrence/death 12 months – recurrence</td>
</tr>
<tr>
<td>Devata et al, 2012(^{29})</td>
<td>1</td>
<td>pNET</td>
<td>Capecitabine plus temozolomide</td>
<td>Partial response. R0 resection</td>
<td>3 months - free of disease</td>
</tr>
<tr>
<td>Barber et al, 2012(^{34})</td>
<td>1/5</td>
<td>pNET</td>
<td>PRCRT ((^{177})Lu-octreotate plus 5-FU)</td>
<td>Partial response. R0 resection</td>
<td>12 months – free of disease</td>
</tr>
<tr>
<td>Lee et al, 2013(^{35})</td>
<td>1/9</td>
<td>pNET</td>
<td>RT</td>
<td>Partial response. R0 resection</td>
<td>5 years – free of disease</td>
</tr>
<tr>
<td>Dumont et al, 2015(^{28})</td>
<td>28/42</td>
<td>pNETs G1/2 with segmental portal hypertension</td>
<td>Chemotherapy (5-FU, streptozocin, doxorubicin, cisplatin, etoposide, oxaliplatin)</td>
<td>No radiological improvement in SPH signs. 13 R0, 6 R1 and 9R2 resections. Incomplete resections due to metastatic disease. All primary tumors resected</td>
<td>5-year overall survival [R0] vs [R1/R2/no resection]; 78% vs 55% (p=0.227)</td>
</tr>
<tr>
<td>Ezzidin et al, 2012(^{32})</td>
<td>1</td>
<td>pNET with liver metastases</td>
<td>PRRT ((^{177})Lu-octreotate)</td>
<td>Partial response. R0 resection, almost complete regression of liver metastases</td>
<td>22 months – complete local remission</td>
</tr>
</tbody>
</table>

\(^{a}\)Patients that underwent surgical exploration following neoadjuvant therapy/patients included in the study.

NECs: neuroendocrine carcinoma(s); pNETs: pancreatic neuroendocrine tumor(s); PRRT: peptide receptor radionuclide therapy; PRCRT: peptide receptor chemoradiation therapy; nr: not reported; RT: radiotherapy.
etoposide and oxaliplatine. No radiological improvement was recorded in segmental portal hypertension signs. Subsequently, 28 of them underwent surgical exploration. Complete resection (R0) was achieved in 13 cases. In 6 patients there was microscopic residual disease (R1 resection) and in the remaining 9 patients there was gross residual disease (R2 resection). It should be underlined that all primary tumors were successfully resected and that incomplete resections were due to intraoperatively found unresectable liver metastases. In survival analysis, a trend towards improved 5-year survival was observed among patients with R0 resections as compared to those with R1/R2 resections and no resection, without yet, reaching statistical significance (78% vs 55% respectively, \( p=0.227 \)).

**BIOLOGICAL AGENTS**

Devata et al reported one patient with pNET who responded partially to the combination of two biological agents, capecitabine plus temozolomide, and underwent R0 resection. The patient remained alive and free of disease 3 months postoperatively.

**PEPTIDE RECEPTOR RADIONUCLIDE THERAPY**

Six studies have reported successful use of peptide receptor radionuclide therapy in the neoadjuvant setting. Kwekkeboom et al retrospectively studied 310 patients with pNETs who received PRRT with \(^{177}\)Lu-octreotate. Four of them, with non-functioning pNETs that responded partially, underwent R0 resection. One of them died postoperatively from surgical complications.

Kaemmerer et al reported a patient with PNEC who responded partially to PRRT with \(^{90}\)Y-DOTA-TATE and was subsequently completely resected. The patient was alive and free of disease 18 months after the operation.

Stoeltzing et al reported another patient with resected pNET and bilobular liver metastases which were successfully resected after partial response to PRRT with \(^{90}\)Y-DOTA-TOC. The patient was alive and free of disease 12 months after the operation.

Ezzidin et al reported a patient with pNET and liver metastases who received neoadjuvant therapy with \(^{177}\)Lu-octreotate demonstrating partial response with tumor shrinkage and one small residual metastatic liver lesion. The primary tumor was then completely resected and the patient remained in complete local remission 22 months after the operation.

In another study, six patients with advanced pNETs were treated with \(^{90}\)Y-DOTA-TATE, two of which underwent surgical exploration following therapy. In one patient the tumor was found to have remained unresectable and an R2 resection was undertaken. In the other one complete response of liver metastases was noted, while the primary tumor was completely resected. Barber et al reported treating five patients with inoperable NETs with the combination of PRRT (\(^{177}\)Lu-octreotate) plus 5-FU. One of them underwent subsequent R0 resection and remained 12 months postoperatively alive and free of disease.

**RADIOThERAPY**

The use of radiotherapy alone in the neoadjuvant setting in advanced pNETs has been described once by Lee et al. Among nine patients who received external beam radiation, only one was offered surgical resection following radiotherapy. Surgical margins were negative and the patient survived 5 years free of disease.

**CONCLUSIONS**

Increasing evidence supports the application of neoadjuvant protocols in advanced pNETs. Patients with pNETs are frequently diagnosed with advanced stage disease and inoperable tumors. Provided that an aggressive surgical approach is indicated in patients with pNETs, efforts to downsize locally advanced tumors and make them resectable seem perfectly reasonable.

Several preoperative therapies have been suggested in the literature, including chemotherapy, radiotherapy, biological agents, peptide receptor radionuclide therapy or various combinations of them.

Neuroendocrine tumors are relatively rare tumors, with most of the available evidence deriving from case reports or small case series treating heterogeneous
Neoadjuvant therapy for advanced pancreatic neuroendocrine tumors

21

tumors. The latter is due to the fact that prospective and controlled randomized clinical trials from high-volume institutions are not feasible. Expert panel consensus based on the experience of surgeons and endocrinologists who deal with locally advanced pancreatic neuroendocrine tumors, is needed to assess the efficacy and survival benefit of the aforementioned neoadjuvant treatments and define the optimal treatment algorithm. Treatment recommendation for pNETs may not strictly follow the current guidelines and must include individualization and optimization of management.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest and that they received no specific funding for this article.

REFERENCES

23. Ferrone CR, Marchegiani G, Hong TS, et al, 2015 Radiological and surgical implications of neoadjuvant treatment with FOLFIRINOX for locally advanced and
22. I. Perysinakis ET AL


24. Kaemmerer D, Prasad V, Daffner W, et al, 2009 Neo-
adjuvant peptide receptor radionuclide therapy for an

after neoadjuvant chemotherapy in metastatic poorly

after neoadjuvant chemotherapy for locally advanced
extrapulmonary poorly differentiated neuroendocrine

27. Sato I, Ueda N, Kinoshita E, et al, 2010 Curatively
resected case of non-functioning pancreatic neuroen-
docrine carcinoma with multiple liver metastases after
downstaging with S-1 monotherapy. Gan To Kagaku Ryoho 37: 1341-1344.

E, Elias D, 2015 Therapeutic strategies for advanced

with capecitabine and temozolomide for unresectable

Treatment with the radiolabeled somatostatin analog

surgery with neoadjuvant 90Y-DOTATOC therapy for
down-sizing synchronous bilobular hepatic metastases

Neoadjuvant downsizing by internal radiation: a case
for preoperative peptide receptor radionuclide therapy

Peptide receptor radionuclide therapy as a potential
tool for neoadjuvant therapy in patients with inoperable

34. Barber TW, Hofman MS, Thomson BN, Hicks RJ, 2012
The potential for induction peptide receptor chemo-
radiomunocle therapy to render inoperable pancreatic

35. Lee J, Choi J, Choi C, Seong J, 2013 Role of radio-
therapy for pancreatobiliary neuroendocrine tumors.
Radiat Oncol J 31: 125-130.
Recent advances in the molecular mechanisms causing primary generalized glucocorticoid resistance

Nicolas C. Nicolaides,1,2 Agaristi Lamprokostopoulou,2 Amalia Sertedaki,1 Evangelia Charmandari1,2

1Division of Endocrinology, Metabolism and Diabetes, First Department of Pediatrics, University of Athens Medical School, ‘Aghia Sophia’ Children’s Hospital; 2Division of Endocrinology and Metabolism, Center of Clinical, Experimental Surgery and Translational Research, Biomedical Research Foundation of the Academy of Athens; Athens, Greece

ABSTRACT
Primary Generalized Glucocorticoid Resistance is a rare condition characterized by generalized, partial, target tissue insensitivity to glucocorticoids owing to inactivating mutations, insertions or deletions in the human glucocorticoid receptor (hGR) gene (NR3C1). Recent advances in molecular and structural biology have enabled us to elucidate the molecular mechanisms of action of the mutant receptors and to understand how certain conformational alterations of the defective hGRs result in generalized glucocorticoid resistance. Furthermore, our ever-increasing understanding of the molecular mechanisms of glucocorticoid action indicates that the glucocorticoid signaling pathway is a stochastic system that plays a fundamental role in maintaining both basal and stress-related homeostasis. In this review, we summarize the clinical manifestations and molecular pathogenesis of Primary Generalized Glucocorticoid Resistance, we present our recent findings from the functional characterization of three novel heterozygous point mutations in the NR3C1 gene, and we discuss the diagnostic approach and therapeutic management of the condition. When the condition is suspected, we recommend sequencing analysis of the NR3C1 gene as well as of other genes encoding proteins involved in the glucocorticoid signal transduction. The tremendous progress of next-generation sequencing will undoubtedly uncover novel hGR partners or cofactors.

Key words: Glucocorticoids, Glucocorticoid receptor, Glucocorticoid resistance, Glucocorticoid signaling, NR3C1 mutations

INTRODUCTION
Glucocorticoids (cortisol in humans, corticosterone in most rodents) are steroid hormones secreted by the adrenal cortex into the systemic circulation in an ultradian, circadian, and stress-related fashion under the control of the hypothalamic-pituitary-adrenal (HPA) axis.1-4 These cholesterol-derived molecules
participate in the physiologic function of almost all organs and play a fundamental role in the stress response. Glucocorticoids exert their pleiotropic effects through their cognate receptor, which belongs to the steroid receptor family of the nuclear receptor superfamily. The glucocorticoid receptor functions as a ligand-activated transcription factor that influences the transcription rate of numerous genes through well-described genomic and less well understood nongenomic actions. Since glucocorticoids contribute substantially to the steady state of the organism, it is generally accepted that glucocorticoid signaling is not merely a simplified signal transduction pathway but a complex homeostatic system that functions coordinately with other systems to help the organism cope with stressful stimuli.

Homeostatic mechanisms, including the HPA axis, exert their effects in an inverted U-shaped dose-response curve (Figure 1). Normal basal homeostasis or eustasis is achieved in the central, optimal range of the curve, whereas suboptimal effects may occur on either side of the curve and can lead to insufficient adaptation, a state that has been called allostasis or cacostasis. The latter states of hypofunction or hyperfunction of the HPA axis may have short-term or long-term adverse consequences for the individual and can lead to a compromised sense of well-being and/or performance. At the molecular level, any alterations in the glucocorticoid signal transduction are likely to result in impaired tissue sensitivity to glucocorticoids, which may present with clinical manifestations of glucocorticoid resistance or glucocorticoid hypersensitivity (Table 1). One such allostatic condition is Primary Generalized Glucocorticoid Resistance.

**PATHOPHYSIOLOGY AND CLINICAL MANIFESTATIONS**

Primary Generalized Glucocorticoid Resistance is a rare familial or sporadic allostatic condition in which almost all organs have a different degree of insensitivity to glucocorticoids. This decreased tissue responsiveness to glucocorticoids leads to compensatory activation of the HPA axis that causes hypersecretion of adrenocorticotropic hormone (ACTH) by the anterior pituitary gland. The increased concentrations of ACTH cause adrenal cortex hypertrophy and activate the enzymatic biosynthetic pathway of cortisol, adrenal androgens [androstenedione, dehydroepiandrosterone (DHEA), and DHEA-sulfate (DHEAS)], and steroid precursors with mineralocorticoid activity (deoxycorticosterone and corticosterone).

Patients with Primary Generalized Glucocorticoid

<table>
<thead>
<tr>
<th>Target tissue</th>
<th>Glucocorticoid hypersensitivity = Glucocorticoid excess</th>
<th>Glucocorticoid resistance = Glucocorticoid deficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central nervous system</td>
<td>Insomnia, anxiety, depression, defective cognition</td>
<td>Fatigue, somnolence, malaise, defective cognition</td>
</tr>
<tr>
<td>Liver</td>
<td>+ Gluconeogenesis, + lipogenesis</td>
<td>Hypoglycemia, resistance to diabetes mellitus</td>
</tr>
<tr>
<td>Fat</td>
<td>Accumulation of visceral fat (metabolic syndrome)</td>
<td>Loss of weight, resistance to weight gain</td>
</tr>
<tr>
<td>Blood vessels</td>
<td>Hypertension</td>
<td>Hypotension</td>
</tr>
<tr>
<td>Bone</td>
<td>Stunted growth, osteoporosis</td>
<td></td>
</tr>
<tr>
<td>Inflammation/immunity</td>
<td>Immune suppression, anti-inflammation, vulnerability to certain infections and tumors</td>
<td>+ Inflammation, + autoimmunity, + allergy</td>
</tr>
</tbody>
</table>

Figure 1. The inverted U-shaped dose-response curve. Eustasis is achieved in the middle range of the homeostatic system activity, whereas allostasis or cacostasis occurs when the homeostatic system activity is deficient or excessive.
Resistance may be asymptomatic or may present with clinical manifestations of mineralocorticoid and/or androgen excess. Therefore, hypertension and/or hypokalemic alkalosis can occur in patients with increased concentrations of steroid precursors with mineralocorticoid activity.11-18 Adrenal androgen excess may cause ambiguous genitalia in karyotypic females, precocious puberty, acne, hirsutism, male-pattern hair loss, and hypofertility in both sexes, oligo-amenorrhea and menstrual irregularities in women, and oligospermia in men.11-18 Glucocorticoid deficiency is rare and has been reported in adults with chronic fatigue,16,19,20 in a child with hypoglycemic generalized tonic-clonic seizures during an episode of febrile illness,21 and in a newborn with profound hypoglycemia, reported easy “fatigability” with feeding, and growth hormone deficiency.22 It is worth noting that the increased CRH concentrations may cause anxiety and depression.18

The clinical heterogeneity of the condition is mostly due to differences in target tissue sensitivity to glucocorticoids, mineralocorticoids, and adrenal androgens among patients.11-18 Furthermore, other molecules participating in steroid signaling pathways, such as hormone inactivating or -activating enzymes, immunophilins, and heat shock proteins, as well as genetic and epigenetic factors, may contribute substantially to variations in tissue response to steroid hormones.15,17,18

**MOLECULAR PATHOGENESIS**

In Generalized Glucocorticoid Resistance, the decreased target-tissue sensitivity to glucocorticoids has been primarily ascribed to inactivating point mutations, insertions or deletions in the NR3C1 gene, which encodes the human glucocorticoid receptor (hGR).11-18 For many years it was believed that the NR3C1 gene encoded one protein. During the last three decades, this classic dogma changed dramatically with the demonstration that the alternative use of exon 9α or 9β of the NR3C1 gene upon transcription generates the two main protein isoforms, the hGRα and the hGRβ, which have different properties in terms of localization, ligand-binding ability, and transcriptional activity.23-27 Moreover, Lu and Cidlowski showed that the hGRα mRNA may be translated into eight receptor α isoforms (hGRα-A, hGRα-B, hGRα-C1, hGRα-C2, hGRα-C3, hGRα-D1, hGRα-D2, and hGRα-D3) because of the presence of eight alternative translation initiation sites.28,29 It is likely that the hGRβ mRNA may also be translated into eight receptor β isoforms through the same molecular mechanisms.

The classic hGRα is a modular protein that consists of four functional domains: i) the amino-terminal or immunogenic domain (NTD), which is the largest domain of the receptor and consists of amino acids that undergo several post-translational modifications; ii) the DNA-binding domain (DBD), which contains the conserved motif of two zinc fingers enabling the receptor to bind to DNA sequences within the promoter regions of glucocorticoid-responsive genes; iii) the hinge region, which confers the appropriate structural flexibility to the receptor and contains critical lysine residues that undergo acetylation by the transcription factor CLOCK, the circadian locomotor output cycle kaput which, together with the brain-muscle-arnt-like protein 1 (BMAL1), regulate the circadian oscillations of gene expression; and iv) the ligand-binding domain (LBD), where the receptor binds to natural or synthetic glucocorticoids.5,6,18 The LBD consists of twelve α helices and four β sheets and contains amino acid sequences important for the ligand-induced nuclear translocation of the receptor, as well as amino acids that interact with coactivators or corepressors in a ligand-dependent fashion.5,6,18

At the target cell, the glucocorticoid signaling cascade is triggered upon glucocorticoid-binding to the LBD of the receptor and leads to conformational changes that result in dissociation of the receptor from heat shock proteins and immunophilins (Figure 2).5,6,18 The ligand-bound hGRα translocates into the nucleus, forms homo- or hetero-dimers, and binds to the specific DNA sequences, the glucocorticoid response elements (GREs), within the regulatory regions of glucocorticoid target genes, thereby inducing or repressing their expression. Alternatively, the activated hGRα can influence gene expression independently of DNA binding by physically interacting with other important transcription factors, such as the nuclear factor-κB (NF-κB), the activator protein-1 (AP-1), and signal transducers and activators of transcription (STATs) (Figure 2).5,6,18
Figure 2. The HPA axis and the glucocorticoid signaling pathway. Upon stimulation of the HPA axis by numerous external or internal stressful stimuli, neurons of the paraventricular nuclei located in the hypothalamus release CRH and AVP, which both increase the production and secretion of ACTH by the anterior lobe of pituitary gland. ACTH then triggers the production of glucocorticoids, which reach every target-cell through the systemic circulation. In the target-cell, glucocorticoids bind to their cognate receptor which undergoes conformational changes, dissociates from HSPs and FKBP, and translocates to the nucleus, where it binds as a homo- or heterodimer onto the GREs of target-genes, thereby inducing or repressing the expression of the latter. The hGR can alternatively regulate gene expression, independently of DNA binding, by physically interacting with other transcription factors (NF-κB, AP-1 or STAT5). Moreover, the hGR was recently shown to undergo acetylation by the transcription factor CLOCK in a lysine cluster of its hinge region. This CLOCK-mediated post-translational modification of the hGR may provide the basis for the circadian oscillations of glucocorticoid-target genes. In addition to their genomic actions, accumulating evidence suggests that glucocorticoids may induce some effects within seconds or minutes. These nongenomic glucocorticoid actions seem to be mediated by membrane-bound hGRs which activate the mitogen-activated protein kinase (MAPK) or the phosphatidylinositol 3-kinase (PI3K) pathways. ACTH: adrenocorticotropic hormone; AVP: arginine-vasopressin; BMAL1: brain–muscle–arnt-like protein 1; CLOCK: circadian locomotor output cycle kaput; cPLA2α: cytosolic phospholipase A2 alpha; CRH: corticotropin-releasing hormone; eNOS: endothelial nitric oxide synthetase; FKBP: immunophilins; GR: glucocorticoid receptor; HSP: heat shock proteins; MAPK: mitogen-activated protein kinases; NO: nitric oxide; PI3K: phosphatidylinositol 3-kinase; TF: transcription factor.

Primary Generalized Glucocorticoid Resistance is primarily caused by inactivating mostly heterozygous but also homozygous point mutations, insertions or deletions in the NR3C1 gene that lead to a defective glucocorticoid receptor and impaired glucocorticoid signaling and cause generalized, partial tissue in-
sensitivity to glucocorticoids. Most of the reported NR3C1 gene mutations are located in the LBD of the receptor, three of them, however, the hGRαV423A, the hGRαR469X and the hGRαR477H, having been identified in the DBD (Figure 3). Over the last three decades, advances in molecular and structural biology have enabled the study of the molecular mechanisms through which the mutant hGRs impair glucocorticoid signal transduction and cause the variable clinical phenotype of Primary Generalized Glucocorticoid Resistance (Table 2).

THE MOLECULAR AND STRUCTURAL BIOLOGY OF THE NATURAL MUTANT RECEPTORS hGRαV423A, hGRαV575G AND hGRαH726R

We have recently identified three novel heterozygous inactivating point mutations in the NR3C1 gene causing Primary Generalized Glucocorticoid Resistance and we have applied standard molecular and structural biology methods to elucidate the molecular mechanisms of action of the mutant receptors. Specifically, we investigated: i) the ability of the mutant receptors to induce glucocorticoid-responsive genes through reporter assays; ii) the expression of the mutant receptors at the protein level via Western blotting; iii) the ability of the mutant receptors to exert a dominant negative effect upon the hGRα-mediated transcriptional activity using reporter assays; iv) the transrepressive activity of the mutant receptors upon the NF-κB-mediated transcriptional activity through reporter assays; v) the affinity of the mutant receptors for the ligand via dexamethasone-binding assays; vi) the subcellular localization of the mutant receptors in the absence of ligand and the time required to complete nuclear translocation following exposure to dexamethasone using green fluorescent protein (GFP)-fused plasmids; vii) the binding of the mutant receptors to GREs through in vitro binding assays; viii) the ability of the mutant receptors to interact with coactivators, such as the glucocorticoid receptor-interacting protein 1 (GRIP1) coactivator, using Glutathione-S-Transferase (GST)-pull down assays; and ix) the conformational changes of the mutant receptors causing Primary Generalized Glucocorticoid Resistance through computer-based 3-dimensional simulation using crystallographic data available in public.

The first patient was a 9-year-old boy who presented with anxiety, fatigue, and hypertension. He harbored a novel heterozygous mutation in the NR3C1 gene that resulted in substitution of valine (V) by alanine (A) at amino acid position 423 in the LBD of the receptor. In vitro functional studies showed that

Figure 3. Schematic representation of the known mutations of the NR3C1 gene causing Primary Generalized Glucocorticoid Resistance. Mutations in the upper panel are located in the LBD of the receptor, while the V423A, R469X, and R477H mutations are located in the DBD of the receptor.
Table 2. Mutations of the human glucocorticoid receptor gene causing Primary Generalized Glucocorticoid Resistance

<table>
<thead>
<tr>
<th>Author (Reference)</th>
<th>Mutation position</th>
<th>Molecular mechanisms</th>
<th>Genotype</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chrousos et al19</td>
<td>1922 (A→T) 641 (D→V)</td>
<td>Transactivation ↓ Affinity for ligand ↓ (x 3) Nuclear translocation: 22 min Abnormal interaction with GRIP1</td>
<td>Homozygous</td>
<td>Hypertension Hypokalemic alkalosis</td>
</tr>
<tr>
<td>Hurley et al32</td>
<td>4 bp deletion in exon-intron 6</td>
<td>Inactivation of the affected allele</td>
<td>Heterozygous</td>
<td>Hirsutism Male-pattern hair-loss Menstrual irregularities</td>
</tr>
<tr>
<td>Karl et al33</td>
<td>2185 (G→A) 729 (V→I) 4 bp deletion in exon-intron 6</td>
<td>Transactivation ↓ Affinity for ligand ↓ (x 2) Nuclear translocation: 120 min Abnormal interaction with GRIP1</td>
<td>Heterozygous</td>
<td>Precocious puberty Hyperandrogenism</td>
</tr>
<tr>
<td>Malchoff et al34</td>
<td>1676 (T→A) 559 (I→N)</td>
<td>Transactivation ↓ Decrease in hGR binding sites Transdominance (+) Nuclear translocation: 180 min Abnormal interaction with GRIP1</td>
<td>Heterozygous</td>
<td>Hypertension Oligospermia Infertility</td>
</tr>
<tr>
<td>Charmandari et al39</td>
<td>1430 (G→A) 477 (R→H)</td>
<td>Transactivation ↓ No DNA binding Nuclear translocation: 20 min</td>
<td>Heterozygous</td>
<td>Hirsutism Fatigue Hypertension</td>
</tr>
<tr>
<td>Karl et al31</td>
<td>2035 (G→A) 679 (G→S)</td>
<td>Transactivation ↓ Affinity for ligand ↓ (x 2) Nuclear translocation: 30 min Abnormal interaction with GRIP1</td>
<td>Heterozygous</td>
<td>Hirsutism Fatigue Hypertension</td>
</tr>
<tr>
<td>Charmandari et al40</td>
<td>1712 (T→C) 571 (V→A)</td>
<td>Transactivation ↓ Affinity for ligand ↓ (x 6) Nuclear translocation: 25 min Abnormal interaction with GRIP1</td>
<td>Homozygous</td>
<td>Ambiguous genitalia Hypertension Hypokalemia Hyperandrogenism</td>
</tr>
<tr>
<td>Vottero et al38</td>
<td>2241 (T→G) 747 (I→M)</td>
<td>Transactivation ↓ Transdominance (+) Affinity for ligand ↓ (x 2) Nuclear translocation ↓ Abnormal interaction with GRIP1</td>
<td>Heterozygous</td>
<td>Cystic acne Hirsutism Oligo-amenorrhea</td>
</tr>
<tr>
<td>Charmandari et al40</td>
<td>2318 (T→C) 773 (L→P)</td>
<td>Transactivation ↓ Transdominance (+) Affinity for ligand ↓ (x 2.6) Nuclear translocation: 30 min Abnormal interaction with GRIP1</td>
<td>Heterozygous</td>
<td>Fatigue Anxiety Acne Hirsutism Hypertension</td>
</tr>
<tr>
<td>Charmandari et al32</td>
<td>2209 (T→C) 737 (F→L)</td>
<td>Transactivation ↓ Transdominance (+) Affinity for ligand ↓ (x 1.5) Nuclear translocation: 180 min</td>
<td>Heterozygous</td>
<td>Hypertension Hypokalemia</td>
</tr>
<tr>
<td>McMahon et al32</td>
<td>2 bp deletion at nt 2318-9 773</td>
<td>Transactivation ↓ Affinity for ligand: absent No suppression of IL-6</td>
<td>Homozygous</td>
<td>Hypoglycemia Fatigability with feeding Hypertension</td>
</tr>
</tbody>
</table>
Table 2. (continued) Mutations of the human glucocorticoid receptor gene causing Primary Generalized Glucocorticoid Resistance

<table>
<thead>
<tr>
<th>Author (Reference)</th>
<th>Mutation position</th>
<th>cdNA</th>
<th>Amino acid</th>
<th>Molecular mechanisms</th>
<th>Genotype</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nader et al21</td>
<td>2141 (G→A) 714 (R→Q)</td>
<td>Transactivation ↓</td>
<td>Heterozygous</td>
<td>Hypoglycemia, Hypokalemia, Hypertension, Mild clitoromegaly, Advanced bone age, Precocious puberty</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transdominance (+)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Affinity for ligand ↓ (x 2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nuclear translocation ↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Abnormal interaction with GRIP1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bouligand et al43</td>
<td>1405 (C→T) 469 (R→X)</td>
<td>Transactivation ↓</td>
<td>Heterozygous</td>
<td>Adrenal hyperplasia, Hypertension, Hypokalemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ligand-binding sites ↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>No DNA binding</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>No nuclear translocation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zhu Hui-juan et al44</td>
<td>1667 (G→T) 556 (T→I)</td>
<td>Not studied yet</td>
<td>Heterozygous</td>
<td>Adrenal incidentaloma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Roberts et al45</td>
<td>1268 (T→C) 423 (V→A)</td>
<td>Transactivation ↓</td>
<td>Heterozygous</td>
<td>Fatigue, Anxiety, Hypertension</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Affinity for ligand: N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>No DNA binding</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nuclear translocation: 35 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Interaction with GRIP1: N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicolaides et al46</td>
<td>1724 (T→G) 575 (V→G)</td>
<td>Transactivation ↓</td>
<td>Heterozygous</td>
<td>Melanoma, Asymptomatic daughters</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transrepression ↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Affinity for ligand ↓ (x 2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nuclear translocation ↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Abnormal interaction with GRIP1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicolaides et al47</td>
<td>2177 (A→G) 726 (H→R)</td>
<td>Transactivation ↓</td>
<td>Heterozygous</td>
<td>Hirsutism, Acne, Alopecia, Anxiety, Fatigue, Irregular menstrual cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transrepression ↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Affinity for ligand ↓ (x 2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nuclear translocation ↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Abnormal interaction with GRIP1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

the hGRαV423A displayed reduced transcriptional activity, had a significant reduction in its ability to bind to DNA sequences within the promoter regions of glucocorticoid-target genes, and required a longer time to translocate into the nucleus following exposure to dexamethasone, compared with the wild-type receptor (Figure 4A and 4B).45 Structural biology studies highlighted the critical role of the hydrophobic valine at this position within the first zinc finger of the DBD of the receptor. The hydrophobic nature of valine at amino acid position 423 protects the four zinc-binding cysteines (C421, C424, C438, and C441) from the destructive diffusion of water molecules. The substitution of valine by alanine results in water diffusion into the ion-binding region of the mutant receptor and causes reduced binding of the mutant receptor hGRαV423A to GREs.45

The second mutation in the NR3C1 gene was identified in a 70-year-old man and his two daughters, who had increased urinary free cortisol excretion and showed resistance of the HPA axis to dexamethasone suppression without any symptoms or signs suggestive of Cushing syndrome.46 Sequencing of the NR3C1 gene revealed a substitution of valine (V) by glycine (G) at amino acid 575 in the LBD of the receptor.46 Compared with the wild-type receptor, the hGRαV575G had 50% lower affinity for dexamethasone, displayed reduced transactivation of glucocorticoid-responsive genes, had a 2.5-fold delay in nuclear translocation, and interacted with the GRIP1 coactivator mostly through its AF-1
domain (Figure 4C).\textsuperscript{46} This impaired interaction of the mutant receptor with the GRIP1 coactivator was further confirmed by structural biology assays which showed that the substitution of valine by glycine at amino acid position 575 resulted in the loss of two noncovalent bonds observed between the valine of the wild-type receptor and the LXXLL motif of the GRIP1 coactivator. Finally, the hGRαV575G demonstrated significantly increased ability to transrepress NF-κB-responsive genes (Figure 4C).\textsuperscript{46}

The last point mutation in the \textit{NR3C1} gene was identified in a 30-year-old woman with hirsutism, acne, alopecia, anxiety, fatigue, and irregular menstrual cycles without any clinical features of Cushing syndrome.\textsuperscript{47} Endocrinologic evaluation revealed elevated 08:00 h plasma ACTH, serum cortisol concentrations, and increased urinary free cortisol (UFC) excretion. There was resistance of the HPA axis to overnight dexamethasone suppression, while a pituitary magnetic resonance imaging scan was normal.\textsuperscript{47}

\textbf{Figure 4.} Molecular mechanisms of action of the mutant receptors hGRαV423A, hGRαV575G, and hGRαH726R, compared with the wild-type hGRα. (A) The hGRαWT-mediated signal transduction. (B) Molecular mechanisms of action of the hGRαV423A. (C) Molecular mechanisms of action of the hGRαV575G. (D) Molecular mechanisms of action of the hGRαH726R. FKBP: immunophilins; GRIP1: glucocorticoid receptor-interacting protein 1; H726R: human glucocorticoid receptor H726R; HSP: heat shock proteins; p50: transcription factor p50; p65: transcription factor p65; V423A: human glucocorticoid receptor V423A; V575G: human glucocorticoid receptor V575G; WT: wild-type human glucocorticoid receptor.
A novel heterozygous point mutation was identified in the NR3C1 gene, which resulted in histidine (H) to arginine (R) substitution at amino acid position 726 in the LBD of the receptor. We subsequently elucidated the molecular mechanisms of action of the mutant receptor hGRαH726R causing Primary Generalized Glucocorticoid Resistance. The hGRαH726R displayed reduced ability to transactivate target genes and to transrepress NF-κB-responsive genes, had 55% lower affinity for the ligand and a 4-fold delay in cytoplasmic-to-nuclear translocation following dexamethasone-induced activation, and interacted with the GRIP1 coactivator mostly through its AF-1 domain (Figure 4D). Structural biology studies showed that the H726R mutation caused a structural shift in the rigidity of helix 10 within the LBD of the receptor, which resulted in reduced flexibility and decreased affinity of the mutant receptor for the ligand.

**DIAGNOSIS**

When Primary Generalized Glucocorticoid Resistance is suspected, a detailed personal and family history should be obtained, placing particular emphasis on any clinical manifestations indicating alterations in the activity of the HPA axis. Symptoms such as seizures, headaches or visual impairment should be carefully evaluated. The irregularity of menstrual cycles in women should be methodically documented. Furthermore, the growth, development and pubertal stage in children should be assessed. On clinical examination, particular attention should be paid to signs suggestive of mineralocorticoid and/or androgen excess.

The endocrinologic evaluation includes determination of the 08:00h concentrations of serum cortisol, plasma ACTH, plasma renin activity (recumbent), serum aldosterone, androgens (testosterone, androstenedione, DHEA, DHEAS), and insulin. The biochemical evaluation consists of measurement of the 08:00h concentrations of total cholesterol, HDL, LDL, triglycerides, and fasting glucose. Patients with Primary Generalized Glucocorticoid Resistance have increased 24-hour serum cortisol concentrations and elevated 24-hour UFC excretion despite the absence of Cushingoid features; therefore, the 24-h UFC excretion should be determined on 2 or 3 consecutive days to enable accurate diagnosis of the condition. It is also important to note that patients may display significant variations in the 24-hour UFC excretion and serum cortisol concentrations owing to variations in the impairment of glucocorticoid signal transduction. Indeed, serum cortisol concentrations may be up to 7-fold higher compared with the highest value of its normal range, while the 24-hour UFC excretion may be up to 50-fold higher when compared with the upper normal range. In addition, the 08:00h plasma ACTH concentrations may be normal or high, while the circadian pattern of secretion of both ACTH and cortisol, as well as their responsiveness to any external or internal stressful stimuli, are normal.

The dexamethasone suppression test remains one of the most useful diagnostic tools to evaluate the responsiveness of the HPA axis and to determine the appropriate dose to be administered when treatment is commenced. To this end, increasing doses of dexamethasone (0.3, 0.6, 1.0, 1.5, 2.0, 2.5, 3.0 mg) are administered per os at midnight every other day and serum cortisol and dexamethasone concentrations are determined at 08:00h the following morning. It is also important to determine the serum concentrations of dexamethasone concurrently in order to exclude the possibility of non-adherence to treatment, increased metabolic clearance or reduced absorption of the medication. The HPA axis may display significant variation in its resistance to dexamethasone suppression depending on the impairment of the glucocorticoid signal transduction. The dose of dexamethasone required to suppress serum cortisol concentrations by 50% may be up to 7.5-fold higher than that required to achieve the same degree of HPA axis suppression in normal subjects.

Dexamethasone-binding assays and thymidine incorporation assays remain the two main molecular biology methods that confirm the diagnosis of Primary Generalized Glucocorticoid Resistance. In dexamethasone-binding assays, the administered tritiated dexamethasone binds to the mutated hGR of the patient’s peripheral leukocytes with lower affinity compared with the wild-type hGR of the control subject in patients with mutations in the LBD of the receptor. In thymidine incorporation assays, the patient displays higher resistance to suppression of phytohemagglutinin-stimulated thymidine incorporation in response to dexamethasone compared with
the control subject. Finally, NR3C1 gene insertions, deletions or mutations are identified by sequencing of the coding region (including the intron-exon junctions) of the gene in most but not all subjects with the condition.11-13,15-18

**TREATMENT**

The main aim of treatment in primary generalized glucocorticoid resistance is to suppress the increased secretion of ACTH, thereby suppressing the increased production of adrenal steroids with mineralocorticoid and androgenic activity. Treatment involves administration of high doses of mineralocorticoid-sparing synthetic glucocorticoids, such as dexamethasone (1-3 mg given once daily at night), which activate the mutant and/or wild-type hGRα and suppress the endogenous secretion of ACTH in affected subjects.11-13,15-18 Clinicians should carefully titrate the dose of dexamethasone according to the clinical manifestations and biochemical profile of the patients. It is important to achieve adequate suppression of the HPA axis to prevent the development of an ACTH-secreting adenoma.11-13,15-18

**BEYOND NR3C1 GENE MUTATIONS: PRIMARY GENERALIZED GLUCOCORTICOID RESISTANCE IN THE ERA OF NEXT-GENERATION SEQUENCING**

Although the clinical manifestations of primary generalized glucocorticoid resistance are primarily caused by point mutations, insertions or deletions in the NR3C1 gene encoding defective hGRs, some patients with this condition do not harbor any NR3C1 gene mutations, suggesting a possible role of other genes encoding proteins involved in the glucocorticoid signaling pathway or important hGR partners. One such protein is the FK506-Binding Immunophilin FKBP51, which forms a heterocomplex with the hGRα in the absence of glucocorticoids and is responsible for the cytoplasmic localization of the receptor. Interestingly, some New World primates had elevated expression of FKBP51 and decreased levels of FKBP52, which both contributed to the phenotype of glucocorticoid resistance.48 It was subsequently shown that FKBP51 and FKBP52 have opposite effects in nuclear translocation of GR in mammalian cells, indicating that any imbalance between them could ultimately lead to glucocorticoid resistance or hypersensitivity.49 In addition to the FKBP proteins, the chaperone proteins HSP90 and HSP70 are thought to play a role in determining tissue sensitivity to glucocorticoids. However, their role in glucocorticoid resistance is controversial, given that only a few studies have shown an association between abnormal expression of HSP90/HSP70 and glucocorticoid resistance.50-53

In the era of next-generation sequencing, when a patient is suspected of having primary generalized glucocorticoid resistance, we suggest sequencing of the NR3C1 gene as well as of other genes that encode proteins known to be involved in the glucocorticoid signaling cascade. The application of novel technologies, such as whole-exome sequencing and whole genome sequencing, may uncover other causes of Primary Generalized Glucocorticoid Resistance that may relate to the pathogenesis of this condition.

**FUNDING**

This work was supported by the European Union (European Social Fund – ESF) and Greek national funds through the Operational Program “Education and Lifelong Learning” of the National Strategic Reference Framework (NSRF) – Research Funding Program: THALIS - University of Athens (UOA), Athens, Greece.

**REFERENCES**

6. Nicolaides NC, Galata Z, Kino T, Chrousos GP, Charmandari E, 2010 The human glucocorticoid receptor:
35. Kino T, Stuber RH, Resau JH, Pavlakis GN, Chrousos GP, 2001 Pathologic human GR mutant has a transdomi-
nant negative effect on the wild-type GR by inhibiting its translocation into the nucleus: importance of the ligand-binding domain for intracellular GR trafficking. J Clin Endocrinol Metab 86: 5600-5608.


Lipid accumulation product is associated with metabolic syndrome in women with polycystic ovary syndrome

Djuro Macut,1 Ivana Božić Antić,1 Jelica Bjekić-Macut,2 Dimitrios Panidis,3 Konstantinos Tziomalos,4 Danijela Vojnović Milutinović,5 Olivera Stanojlović,6 Biljana Kastratović-Kotlica,7 Milan Petakov,1 Nataša Milić8

1Clinic for Endocrinology, Diabetes and Diseases of Metabolism, Faculty of Medicine, University of Belgrade; 2CHC Bežanijska kosa, Belgrade, Serbia; 3Division of Endocrinology and Human Reproduction, Second Department of Obstetrics and Gynecology, Aristotle University of Thessaloniki, Hippokration Hospital; 4First Propedeutic Department of Internal Medicine, Aristotle University of Thessaloniki, AHEPA Hospital; Thessaloniki, Greece; 5Department of Biochemistry, Institute for Biological Research “Siniša Stanković”; 6Institute of Medical Physiology, Faculty of Medicine; 7Clinic for Obstetrics and Gynecology, Faculty of Medicine; 8Institute of Medical Statistics, Faculty of Medicine; University of Belgrade, Belgrade, Serbia

ABSTRACT
OBJECTIVE: There is a need for a simple and accurate method for the assessment of cardiovascular risk in polycystic ovary syndrome (PCOS). Lipid accumulation product (LAP) is based on the assessment of waist circumference and serum triglycerides that yield an estimation of lipid overaccumulation. We aimed to determine whether LAP is associated with metabolic syndrome (MetS) in Caucasian women with PCOS. DESIGN: We studied 222 women with PCOS who were diagnosed using the Rotterdam criteria. In all the subjects and controls, LAP was determined and the MetS was assessed using three different international criteria, NCEP-ATP III, IDF, and JIS. ROC curve and logistic regression analyses were performed to determine and analyze associations with the MetS. RESULTS: In the study population the prevalence of MetS was 16.2-19.4%. The cut-off value of 25.9 determined that LAP has the strongest association with MetS whichever international criteria are used, followed by HDL (NCEP-ATP III and JIS) and glucose (IDF). CONCLUSIONS: LAP is used as an independent clinical indicator for MetS in our PCOS women of Caucasian origin. The high diagnostic accuracy of LAP is superseding the need for the use of multiple clinical indicators for the assessment of lipid accumulation as a prerequisite for diagnosis of metabolic and cardiovascular diseases in PCOS women.

Key words: Lipid accumulation product, Metabolic syndrome, Polycystic ovary syndrome, Triglycerides, Waist circumference
INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common endocrinopathy in women of reproductive age, with a prevalence of 6-10%, which is characterized by hyperandrogenic features and chronic oligo-anovulation. Meanwhile, most women with PCOS are also characterized by metabolic abnormalities like insulin resistance, hyperinsulinemia, abdominal obesity, these forming the risk factors for the metabolic syndrome (MetS). Therefore, PCOS is considered to be a metabolic disorder with a number of obesity-related cardiovascular risk factors, specifically insulin resistance, type 2 diabetes, proatherogenic lipid profile, and therefore influencing the susceptibility of those women to develop possible cardiovascular disease later in life.

A significant proportion of women of Caucasian origin with PCOS fulfill the criteria for the MetS irrespectively of the international definition used. However, analyzed PCOS subjects were predominantly overweight or obese. Although excessive weight predominates among PCOS women assessed for the presence of MetS, it is assumed that PCOS women have an increased risk for the metabolic syndrome that is independent of insulin resistance or obesity.

As the diagnosis of MetS is established from the combination of anthropometric and laboratory measures, there is a need for the simplest and the most accurate method of assessment of risk factors to which PCOS women are exposed during the long period of transition from subclinical to overt cardiovascular disease. Therefore, lipid accumulation product (LAP) was proposed in an attempt to develop an easy predictor of cardiovascular disease. This simple clinical index is based on the assessment of waist circumference (WC) and serum triglycerides (TG), yielding an estimation for lipid overaccumulation in adults. LAP was confirmed to be a powerful marker of MetS and diabetes in the general population. Recently, LAP was suggested as being associated with impaired glucose tolerance and MetS in women with PCOS.

The aim of our study was to determine the level of LAP and analyze its association with MetS in a cohort of Caucasian origin women with PCOS.

SUBJECTS AND METHODOLOGY

Subjects

We analyzed 222 women with PCOS (age: 25.01±4.89 years, BMI: 22.99±4.57 kg/m²) and 45 healthy women (age: 28.58±4.91 years, BMI: 21.62±3.88 kg/m²). Subjects were recruited from the outpatient endocrine clinics where they were referred for investigation of oligo- or amenorrhea, fertility problems, hirsutism or acne. PCOS was defined according to the revised 2003 Rotterdam Consensus conference on diagnostic criteria for polycystic ovary syndrome that requires the presence at least two of the following three criteria: (i) oligomenorrhea or anovulation; (ii) clinical and/or biochemical signs of hyperandrogenemia; and (iii) polycystic ovaries on ultrasound. Besides moderate oligo/amenorrhea, our patients had elevated serum testosterone concentrations and appearance of polycystic ovaries. Hyperandrogenemia was defined by serum total testosterone >2 nmol/L, which was based on examination of 56 nonselected women presenting for routine controls who were not hirsute, had regular cycles, and had received no hormonal therapy. None of the examined patients had non-classical 21-hydroxylase deficiency, hyperprolactinemia, Cushing’s disease, impaired fasting glucose (fasting venous glucose ≥6 mmol/L), untreated hypothyroidism or history of drug or alcohol abuse before the diagnosis of PCOS. No patients had received any hormone treatment for at least three months before the study.

The control group consisted of healthy volunteers without any signs of hyperandrogenism, with normal ovulating cycles confirmed by plasma progesterone during the luteal phase of the cycle, and with normal ultrasound appearance of the ovaries.

The study was approved by the Institutional Ethical Committees and written consent was obtained from all subjects.

Methodology

In all subjects, body mass index (BMI), waist circumference (WC), and systolic and diastolic blood pressure (SBP and DBP, respectively) were determined. BMI (kg/m²) was calculated by dividing weight (kg) by height (m) squared, while WC (cm) represented the smallest circumference at the level of the umbilicus.

Baseline blood samples were drawn in all subjects
for determination of total cholesterol (TC), high density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides (TG), fasting plasma glucose (FPG), insulin, testosterone, sex-hormone binding protein (SHBG). All subjects were investigated in the follicular phase of the menstrual cycle (between days 3 and 7), and after a 12h overnight fast. Samples for hormonal analyses were frozen at -80°C until measurement.

Total cholesterol (mmol/L) and triglycerides (mmol/L) were determined using standard enzymatic methods (cholesterol: cholesterol oxidase, Randox, UK; triglycerides: glycerol-3-phosphat oxidase, Randox, UK). HDL (mmol/L) was measured by direct method (Randox, UK), and LDL (mmol/L) determined by the Friedewald formula. Serum insulin (mU/L) concentrations were determined by radioimmunoassay [RIA INSULIN (PEG), INEP, Belgrade, Serbia; intra- and inter-assay CV were 2.5 and 7.7%, respectively]. Serum testosterone (nmol/L) was measured by radioimmunoassay (TESTOCT2, CIS bio international, Gif-Sur-Yvette Cedex, France; intra- and inter-assay CV were 4.5 and 5.1%, respectively). SHBG (nmol/L) was measured by radioimmunoassay (SHBG-RIACT, CIS bio international, Gif-Sur-Yvette Cedex, France; intra- and inter-assay CV were 3.9 and 4.7%, respectively). Free androgen index (FAI) was calculated by the formula [(testosterone × 100) / SHBG] with both testosterone and SHBG expressed in nmol/L. Free androgen index (FAI) >8 was considered as positive for hyperandrogenemia. Insulin resistance was estimated by the homeostasis model assessment of IR (HOMA-IR) method using the formula [HOMA-IR= insulin (mU/L) × glucose (mmol/L)/22.5]. LAP was defined as [(WC - 58) × triglycerides]. The formula includes the minimum WC values used to define sex-specific origin points (58 cm for women) in the Third National Health and Nutrition Examination Survey (NHANES III). Both in PCOS and the controls groups the minimum WC value (60 cm) was quite similar to those used in the original equation for the definition of LAP. The adjustment of the LAP formula according to the minimum WC values of subjects did not change findings (data not shown). For the purpose of comparison we used the original formula.

All subjects were assessed as having MetS using three different international criteria: NCEP-ATP III, IDF, and Joint Interim Statement (JIS) criteria. According to NCEP-ATP III, the diagnosis of MetS was established if any three or more of the following criteria were satisfied: 1) WC: ≥88cm, 2) triglycerides (TG) ≥1.7mmol/L, 3) SBP ≥130 and/or DBP ≥85 mmHg, 4) fasting LDL <1.3mmol/L, and 5) fasting plasma glucose (FPG) ≥6.1 mmol/l. The IDF definition of the MetS for the Europol population considered central adiposity (defined as WC 80 cm) as a prerequisite factor for the diagnosis of the MetS, plus two of the following criteria: TG ≥1.7mmol/L, or specific treatment, low HDL (<1.3mmol/L) or specific treatment, high blood pressure (SBP >130 and/or DBP ≥85mmHg) or treatment of diagnosed hypertension, and FPG ≥5.6mmol/l or type 2 diabetes. According to the JIS criteria, the diagnosis of MetS was established as the presence of any three of the following criteria: 1) central adiposity (WC ≥80cm), 2) TG ≥1.7mmol/L, or specific treatment, 3) HDL <1.3mmol/L or specific treatment, 4) SBP ≥130 and/or DBP ≥85 mmHg or treatment of diagnosed hypertension, and 5) FPG ≥5.6 mmol/l or previously diagnosed type 2 diabetes that is under treatment.

Statistical analyses

Statistical analysis was performed using the Statistical Package for the Social Sciences software (SPSS, version 17.0; SPSS Inc, Chicago, IL, USA). Results are presented as mean ± standard deviation (SD). Normality of data distribution of continuous variables was tested by the Kolmogorov-Smirnov test. In order to achieve normal distribution, all skewed or kurtic data were logarithmically transformed. Differences between groups were analyzed by Student’s T test and univariate analysis of variance (ANOVA) as appropriate. As our women with PCOS were younger than the respective controls, we introduced age as a covariate in all comparisons among groups (ANCOVA). Post hoc Bonferroni adjustment was performed for multiple comparisons. A P value less than 0.05 was considered as statistically significant.

Receiver operating characteristic (ROC) curves were generated for each continuous variable to identify the indicators of MetS defined by NCEP-ATP III, IDF, and JIS criteria. The areas under the curves (AUCs) are provided with standard error of mean (S.E.M.) and 95% confidence intervals (95%CI). ROC curves, a plot of the sensitivity (SEN) (true positive) versus 1-specificity (SP) (false positive) for each potential indicator tested, determine the ability of a screening
measure to correctly identify individuals based on their classification by a reference test. Values for each AUC can be between 0 and 1, with a value of 0.5 indicating that the diagnostic test is no better than chance. We considered that the parameters possessed an accurate diagnostic sensitivity when the AUC value was >0.75.\(^2\) We defined the best cut-off value as the value with the highest proportion of positives and negatives classified correctly by the test. For all variables that are determinants of MetS we used their principal already known cut-off values (or approximate cut-off values if the principal values were not shown) in order to analyze their sensitivity and specificity and compare them to the sensitivity and specificity of the LAP cut-off value. Correlations were analyzed by performing Pearson’s correlation test. Binary logistic regression was performed in order to analyze the determinants of MetS. Univariate logistic regression analyses were performed for LAP and all other known determinants of MetS. All significant determinants from univariate logistic regression analyses were entered into multivariate logistic regression analysis in order to discover indices that were independently associated with MetS in our PCOS population and to analyze if LAP could be one of the established determinants of MetS.

RESULTS

Comparison of clinical characteristics between PCOS and controls and in relation to the presence of MetS

Anthropometric and metabolic characteristics of the women with PCOS and the healthy control women are presented in Table 1. As the PCOS women were younger than the controls, all the comparisons were age adjusted. In comparison to controls, the whole group of PCOS women had significantly higher LAP, WC, DBP, basal insulin and HOMA-IR index, total and LDL cholesterol, triglycerides as well total testosterone and FAI. The prevalence of the MetS according to the NCEP-ATP III, IDF, and JIS criteria for the whole group of PCOS women was 16.2% (36/222), 18.5% (41/222), and 19.4% (43/222), respectively, and for the control group 6.7% (3/45).

In the next step, the women with PCOS were sub-

Table 1. Clinical characteristics of women in PCOS and control groups

<table>
<thead>
<tr>
<th>Analyses</th>
<th>PCOS (N=222)</th>
<th>Controls (N=45)</th>
<th>p</th>
<th>Age adjusted p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>25.01 ± 4.89</td>
<td>28.58 ± 4.91</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.99 ± 4.57</td>
<td>21.62 ± 3.88</td>
<td>0.061</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>78.12 ± 12.77</td>
<td>75.72 ± 10.51</td>
<td>0.258</td>
<td>0.027</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>118.63 ± 12.03</td>
<td>116.67 ± 8.86</td>
<td>0.356</td>
<td>0.128</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>77.73 ± 9.34</td>
<td>75.33 ± 7.64</td>
<td>0.135</td>
<td>0.037</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.66 ± 0.52</td>
<td>4.63 ± 0.50</td>
<td>0.697</td>
<td>0.313</td>
</tr>
<tr>
<td>Insulin (mU/L)</td>
<td>17.71 ± 17.02</td>
<td>13.35 ± 7.37</td>
<td>0.018</td>
<td>0.035</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>3.75 ± 4.12</td>
<td>2.79 ± 1.61</td>
<td>0.022</td>
<td>0.034</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>5.11 ± 1.13</td>
<td>4.86 ± 0.79</td>
<td>0.200</td>
<td>0.017</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.37 ± 0.31</td>
<td>1.47 ± 0.33</td>
<td>0.069</td>
<td>0.024</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>3.20 ± 1.03</td>
<td>2.95 ± 0.70</td>
<td>0.171</td>
<td>0.022</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.17 ± 0.75</td>
<td>0.97 ± 0.65</td>
<td>0.025</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Testosterone (nmol/L)</td>
<td>2.64 ± 1.16</td>
<td>1.79 ± 0.89</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SHBG (nmol/L)</td>
<td>41.02 ± 23.24</td>
<td>62.76 ± 28.19</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FAI (%)</td>
<td>8.70 ± 7.27</td>
<td>3.24 ± 1.37</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LAP</td>
<td>28.94 ± 38.37</td>
<td>18.59 ± 23.47</td>
<td>0.047</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

BMI: body mass index; LAP: lipid accumulation product; WC: waist circumference; SBP: systolic blood pressure; DBP: diastolic blood pressure; HOMA-IR: homeostatic model of insulin resistance; TC: total cholesterol; HDL: high density lipoprotein; LDL: low density lipoprotein; TG: triglycerides; SHBG: sex hormone binding protein; FAI: free androgen index.
Lipid accumulation product and MetS in PCOS women presented in Table 2. Multiple comparisons between PCOS with MetS, PCOS without MetS, and controls were performed for each of the three international criteria used: they showed the same level of significant differences of the analyzed parameters, which did not change after age adjustment (data not shown). LAP was significantly higher in PCOS with MetS in comparison to both PCOS without MetS and controls, classified into six groups related to the presence of MetS using the three international criteria. Because only three women in the control group had MetS, they were not considered for further analyses and the 42 control subjects without MetS were used as a control group for all multiple comparisons. The clinical characteristics of the six groups (PCOS with and without MetS and controls without MetS) are presented in Table 2. Multiple comparisons between PCOS with MetS, PCOS without MetS, and controls were performed for each of the three international criteria used: they showed the same level of significant differences of the analyzed parameters, which did not change after age adjustment (data not shown). LAP was significantly higher in PCOS with MetS in comparison to both PCOS without MetS and controls,

### Table 2. Clinical characteristics of PCOS with and without MetS according to NCEP-ATP III, IDF, and JIS criteria

<table>
<thead>
<tr>
<th>Analyses</th>
<th>Controls (N=42)</th>
<th>PCOS with MetS (N=36)</th>
<th>PCOS without MetS (N=186)</th>
<th>( p )</th>
<th>PCOS with MetS (N=41)</th>
<th>PCOS without MetS (N=181)</th>
<th>( p )</th>
<th>PCOS with MetS-JIS (N=43)</th>
<th>PCOS without MetS-JIS (N=179)</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>28.1±4.4</td>
<td>27.4±6.7</td>
<td>24.5±4.2b</td>
<td>&lt;0.001</td>
<td>27.7±6.6</td>
<td>24.3±4.1b</td>
<td>&lt;0.001</td>
<td>27.7±6.5</td>
<td>24.3±4.13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.1±3</td>
<td>30.3±3.8</td>
<td>21.5±3.3</td>
<td>&lt;0.001</td>
<td>30.1±3.3</td>
<td>21.3±3</td>
<td>&lt;0.001</td>
<td>29.7±3.9</td>
<td>21.3±3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>74.0±8.4</td>
<td>97.1±10.4</td>
<td>74.4±9.6</td>
<td>&lt;0.001</td>
<td>96.2±9.4</td>
<td>73.9±9.4</td>
<td>&lt;0.001</td>
<td>95.1±10.4</td>
<td>73.9±9.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>116.1±8.6</td>
<td>128.1±15.4</td>
<td>116.7±10.3</td>
<td>&lt;0.001</td>
<td>128.6±14.8</td>
<td>116.3±10</td>
<td>&lt;0.001</td>
<td>128.9±14.9</td>
<td>116.1±9.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>4.5±0.4</td>
<td>4.8±0.5</td>
<td>4.6±0.5</td>
<td>0.080</td>
<td>4.9±0.5</td>
<td>4.6±0.4</td>
<td>0.003</td>
<td>4.8±0.5</td>
<td>4.6±0.4</td>
<td>0.004</td>
</tr>
<tr>
<td>Insulin (mU/L)</td>
<td>12.1±4.8</td>
<td>20.5±6.7</td>
<td>17.1±18.3f</td>
<td>&lt;0.001</td>
<td>19.4±7.2</td>
<td>17.3±18.5</td>
<td>0.001</td>
<td>19.1±7.1</td>
<td>17.3±18.6</td>
<td>0.001</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.5±1</td>
<td>4.3±1.4</td>
<td>3.6±4.4f</td>
<td>&lt;0.001</td>
<td>4.1±1.5</td>
<td>3.6±4.5f</td>
<td>&lt;0.001</td>
<td>4.1±1.4</td>
<td>3.6±4.5f</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>4.8±0.7</td>
<td>5.9±1.5</td>
<td>4.9±0.9</td>
<td>&lt;0.001</td>
<td>5.9±1.1</td>
<td>4.9±1</td>
<td>&lt;0.001</td>
<td>6.0±1.4</td>
<td>4.9±0.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.5±0.3</td>
<td>1.0±0.1</td>
<td>1.4±0.3</td>
<td>&lt;0.001</td>
<td>1.0±0.1</td>
<td>1.4±0.3</td>
<td>&lt;0.001</td>
<td>1.0±0.1</td>
<td>1.4±0.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.9±0.6</td>
<td>3.9±1.6</td>
<td>3.0±0.8</td>
<td>&lt;0.001</td>
<td>3.8±1.3</td>
<td>3.0±0.9</td>
<td>&lt;0.001</td>
<td>3.9±1.4</td>
<td>3.0±0.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>0.8±0.3</td>
<td>2.2±1.1</td>
<td>0.9±0.4</td>
<td>&lt;0.001</td>
<td>2.1±1.1</td>
<td>0.9±0.3</td>
<td>&lt;0.001</td>
<td>2.1±1.1</td>
<td>0.9±0.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Testosterone (nmol/L)</td>
<td>1.8±0.8</td>
<td>2.9±1.5</td>
<td>2.5±1b</td>
<td>&lt;0.001</td>
<td>2.9±1.4</td>
<td>2.5±1b</td>
<td>&lt;0.001</td>
<td>2.9±1.4</td>
<td>2.5±1b</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SHBG (nmol/L)</td>
<td>64.4±27.6</td>
<td>26.8±25.1</td>
<td>43.7±21.9b</td>
<td>&lt;0.001</td>
<td>27.8±24.1</td>
<td>43.9±22b</td>
<td>&lt;0.001</td>
<td>28.1±23.6</td>
<td>44.0±22.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FAI (%)</td>
<td>3.1±1.3</td>
<td>15.6±12.6</td>
<td>7.3±4.7</td>
<td>&lt;0.001</td>
<td>15.1±12d</td>
<td>7.2±4.6b</td>
<td>&lt;0.001</td>
<td>14.7±11.8</td>
<td>7.2±4.6b</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LAP</td>
<td>13.4±8.5</td>
<td>91.8±56.7</td>
<td>16.7±15.4</td>
<td>&lt;0.001</td>
<td>87.1±55.1</td>
<td>15.7±14</td>
<td>&lt;0.001</td>
<td>84.4±55.1</td>
<td>15.6±14.1</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>


a = PCOS with MetS vs PCOS without MetS p<0.05; b = PCOS without MetS vs Controls p<0.001; c = PCOS with MetS vs PCOS without MetS p<0.001; d = PCOS with MetS vs Controls p<0.001; e = PCOS with MetS vs Controls p<0.05; f = PCOS without MetS vs Controls p<0.05.
irrespectively of the criteria for the MetS used. All differences are presented in Table 2.

As expected, LAP significantly correlated with BMI, WC, and tryglicerides in all PCOS groups with and without MetS and in controls (data not shown). In PCOS women with MetS, LAP had a significant positive correlation with insulin (NCEP ATP III; IDF and JIS: r=0.391, p=0.025; r=0.470, p=0.003 and r=0.469, p=0.003, respectively), and HOMA-IR (IDF and JIS: r=0.416, p=0.012 and r=0.430, p=0.007, respectively). In PCOS women without MetS, LAP has a significant positive correlation with TC (NCEP-ATP III: r=0.344, p<0.001) and LDL (NCEP-ATP III; IDF and JIS: r=0.354, p<0.001; r=0.314, p<0.001 and r=0.302, p<0.001, respectively), and significant negative correlation with HDL (NCEP-ATP III; IDF and JIS: r= -0.356, p<0.001; r= -0.357, p<0.001 and r= -0.343, p<0.001, respectively).

**Analyses of determinants of MetS in women with PCOS**

By means of the ROC curve analyses, we identified and test diagnostic accuracy of the MetS determinants within our PCOS women and as related to the specific international criteria. The following cut-off values were identified for: LAP 25.9, WC 88.5 cm, BMI 25 kg/m², HDL 1.3 mmol/L, HOMA-IR 3.3, glucose 6.1 mmol/L (for NCEP-ATP III) and 5.6 mmol/L (for IDF and JIS), TG 1.7 mmol/L, SBP 137.5 mmHg, DBP 87.5 mmHg. LAP exhibited high diagnostic accuracy irrespectively of the definition for the MetS used [NCEP-ATP III, IDF and JIS: AUC LAP 0.97 ± 0.01 (95% CI 0.95-0.99), SEN: 81.4%, 93%, 93%, respectively, and SP: 91.3%, 85%, 86%, respectively]. Other predictors for the MetS, namely WC, BMI, HDL cholesterol, and HOMA-IR, are presented in Table 3. Glucose, tryglicerides, systolic and diastolic blood pressure had low diagnostic accuracy for previously defined cut-off values.

LAP cut-off value 25.9 had higher negative predictive value (NPV) than positive predictive value (PPV) irrespectively of the definition for MetS used (NPV for NCEP-ATP III, IDF and JIS: 99.4%, 98%, 98%, respectively, and PPV for NCEP-ATP III, IDF and JIS: 53.8%, 58.5% and 61.5%, respectively).

**Logistic regression**

Univariate binary logistic regression showed that LAP was significantly associated with MetS irrespectively of the international criteria used. In univariate analysis, significant determinants of MetS were also HDL and TG for all international criteria used, while an additional indicator was WC in NCEP-ATP III and JIS, and glucose in IDF and JIS (Table 4).

In order to avoid any problems with multi-collinearity, we omitted WC in IDF from univariate logistic

<table>
<thead>
<tr>
<th>Determinant</th>
<th>NCEP-ATP III</th>
<th></th>
<th></th>
<th>IDF</th>
<th></th>
<th></th>
<th>JIS</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AUC</td>
<td>95% CI</td>
<td>SEN (%)</td>
<td>SP (%)</td>
<td>AUC</td>
<td>95% CI</td>
<td>SEN (%)</td>
<td>SP (%)</td>
<td>AUC</td>
</tr>
<tr>
<td>LAP</td>
<td>0.97±0.01</td>
<td>0.95-0.99</td>
<td>81</td>
<td>91</td>
<td>0.97±0.01</td>
<td>0.95-0.99</td>
<td>93</td>
<td>85</td>
<td>0.97±0.01</td>
</tr>
<tr>
<td>WC</td>
<td>0.94±0.02</td>
<td>0.89-0.98</td>
<td>89</td>
<td>94</td>
<td>0.96±0.01</td>
<td>0.93-0.98</td>
<td>98</td>
<td>83</td>
<td>0.93±0.02</td>
</tr>
<tr>
<td>BMI</td>
<td>0.94±0.03</td>
<td>0.89-0.99</td>
<td>91</td>
<td>87</td>
<td>0.97±0.01</td>
<td>0.94-0.99</td>
<td>93</td>
<td>88</td>
<td>0.94±0.02</td>
</tr>
<tr>
<td>TG</td>
<td>0.93±0.02</td>
<td>0.89-0.97</td>
<td>67</td>
<td>95</td>
<td>0.91±0.03</td>
<td>0.87-0.96</td>
<td>63</td>
<td>96</td>
<td>0.92±0.02</td>
</tr>
<tr>
<td>HDL</td>
<td>0.90±0.02</td>
<td>0.85-0.94</td>
<td>63</td>
<td>97</td>
<td>0.87±0.03</td>
<td>0.81-0.93</td>
<td>63</td>
<td>97</td>
<td>0.87±0.03</td>
</tr>
<tr>
<td>DBP</td>
<td>0.76±0.05</td>
<td>0.66-0.85</td>
<td>33</td>
<td>91</td>
<td>0.76±0.04</td>
<td>0.67-0.85</td>
<td>35</td>
<td>92</td>
<td>0.77±0.04</td>
</tr>
<tr>
<td>SBP</td>
<td>0.75±0.05</td>
<td>0.65-0.85</td>
<td>28</td>
<td>97</td>
<td>0.77±0.05</td>
<td>0.67-0.86</td>
<td>28</td>
<td>97</td>
<td>0.77±0.05</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.73±0.04</td>
<td>0.65-0.81</td>
<td>75</td>
<td>62</td>
<td>0.69±0.05</td>
<td>0.60-0.78</td>
<td>69</td>
<td>62</td>
<td>0.69±0.04</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.59±0.05</td>
<td>0.48-0.69</td>
<td>6</td>
<td>99</td>
<td>0.65±0.05</td>
<td>0.55-0.75</td>
<td>13</td>
<td>98</td>
<td>0.64±0.05</td>
</tr>
</tbody>
</table>

Evaluated by ROC with following cut-off values: LAP: Lipid accumulation product=25.94, WC: Waist circumference=88.5cm for NCEP/ATP III and WC=80.5cm for IDF; BMI: Body mass index=25 kg/m²; TG: Tryglicerides=1.7mmol/L; HDL: High density lipoprotein=1.3mmol/L; DBP: Diastolic blood pressure=87.5mmHg, SBP: Systolic blood pressure=137.5mmHg; HOMA-IR=3.30; Glucose=6.1mmol/L for NCEP/ATP III and glucose=5.6cm for IDF and JIS.
regression analysis and WC and TG (for all international criteria) from multivariate logistic regression analysis. Thus, only LAP and HDL (in NCEP-ATP III), or LAP, HDL, and glucose (in IDF and JIS) entered the analysis performed with blocks (in NCEP-ATP III firstly LAP entered analysis, and then HDL; in IDF and JIS firstly LAP entered analysis, and then HDL and glucose together). In the final model, LAP and HDL remained significantly associated with MetS defined by NCEP-ATP III and JIS criteria, while LAP, HDL, and glucose remained significantly associated with MetS defined by IDF criteria (Table 4). LAP was a more potent indicator than either HDL or glucose in respective international criteria used, but models were stronger when all parameters were present.

DISCUSSION

The results of our study confirmed for the first time a strong association between lipid accumulation product and metabolic syndrome in a selected Europid population of women with PCOS. We found the highest diagnostic accuracy for LAP among other known or related determinants for the MetS and irrespectively of the three international criteria (NCEP-ATP III, IDF or JIS) used.

MetS has a varying frequency of up to 50% among PCOS women, this being mainly related to geographical and racial differences. Prevalence of the MetS using three criteria within our PCOS women was in the range of 16.2 to 19.4% and is similar to the prevalence in other Europid populations. There is a universally established relationship obesity and the MetS. The prevalence of obesity is rising worldwide and is considered to be contributing directly to the current high prevalence of the MetS. It is assumed that the increased prevalence of abdominal obesity and MetS among women with PCOS is directly linked and that even after adjustment for BMI, PCOS did not persist as an independent indicator of MetS in those women. On the other hand, obesity denotes excess fat with consequent dysfunctions that are related to the anatomical regions. In 2005, Henry Kahn proposed a simple index named lipid accumulation index, or LAP, based on the measurement of WC, an indicator of intra-abdominal fat depots, and the fasting concentration of triglycerides, a marker of circulating lipoprotein content. Hence, LAP expresses a continuous metabolic and cardiovascular risk function associated with lipid overaccumulation in adults. Besides the confirmed diagnostic accuracy of LAP in predicting MetS among non-diabetic adults in a Europid population, in PCOS women this novel index was shown to be associated with HOMA index and impaired glucose tolerance, mostly in Caucasian women, and MetS was defined by IDF criteria in

### Table 4. Determinants of MetS in PCOS using different international criteria

<table>
<thead>
<tr>
<th>Variable</th>
<th>NCEP/ATP III</th>
<th>IDF</th>
<th>JIS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RR</td>
<td>95%CI</td>
<td>p</td>
</tr>
<tr>
<td>LAP</td>
<td>182.00</td>
<td>24.00-1379.96</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WC</td>
<td>159.50</td>
<td>42.66-596.33</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL</td>
<td>0.02</td>
<td>0.002-0.116</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TG</td>
<td>35.20</td>
<td>13.73-90.23</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.46</td>
<td>0.74-40.09</td>
<td>0.096</td>
</tr>
<tr>
<td>LAP</td>
<td>92.01</td>
<td>11.82-716.11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL</td>
<td>0.05</td>
<td>0.01-0.37</td>
<td>0.004</td>
</tr>
<tr>
<td>Glucose</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>R²</td>
<td>85.94</td>
<td>103.57</td>
<td>101.39</td>
</tr>
</tbody>
</table>

Evaluated by binary logistic regression with following cut-off values: LAP: Lipid accumulation product=25.94, WC: Waist circumference=88cm for NCEP/ATP III and WC=80cm for IDF; HDL=1.3mmol/L; glucose=6.1mmol/L for NCEP/ATP III and glucose= 5.6cm for IDF and JIS. NA: not analysed.
Chinese women.\textsuperscript{16} However, a study by Xiang et al is the only one demonstrating the relation between LAP and MetS in PCOS, and even though without data for the respective controls, this could account for the high prevalence of MetS shown in this selected Chinese population.\textsuperscript{16} Our mean value for LAP of 28.9 in the whole group of PCOS women is similar to the values shown in the other Europid-based study on PCOS women.\textsuperscript{15} When we used the cut-off value of 25.9 for LAP in the multivariate analyses for each of the three international criteria for the MetS used, the same five indicators for the MetS were fully validated. We showed for the first time in the selected Europid population of women with PCOS that LAP among assessed variables had the strongest association with MetS, and irrespectively of the international definition used (AUC\textsubscript{LAP} 0.97 ± 0.01, respectively). Other indices of the MetS from the ROC analyses, namely WC, BMI, HDL, and HOMA-IR, had lower diagnostic accuracy (Table 3).

The second highest diagnostic accuracy was shared between WC and BMI. It was considered with regard to Europid females that central obesity is present when WC is ≥80 cm,\textsuperscript{29} and that it is related to BMI ≥25 kg/m\textsuperscript{2}.\textsuperscript{34} Moreover, in comparison to BMI, which can often mask remarkable heterogeneity among subjects of similar BMI values, WC assessment provides an effective measure of visceral fat and, if conjuncted with fasting triglyceridemia, represents a useful marker of visceral fat accumulation.\textsuperscript{35} If we take into consideration that BMI was not shown to be an accurate predictor of the risk of obesity-related diseases,\textsuperscript{36} our results are in line with other studies that found WC to be an important clinical predictor of the MetS in respective PCOS populations.\textsuperscript{10,16} Our results, obtained three international criteria for the MetS, are in line with the results of other authors attributing to the WC a high accuracy of prediction of MetS irrespectively of the cut-off values for WC. Although our data showed that WC and BMI shared the same level of accuracy, WC had higher sensitivity in predicting MetS, particularly when the IDF and JIS criteria are used. Moreover, our results on prediction power of WC, using the cut-off value of 80 cm as obligatory or not obligatory in the IDF and JIS criteria for the definition of MetS, supported the view that accepted WC for Europid women\textsuperscript{29} is more appropriate for our group of Caucasian-origin PCOS women.

Low HDL-cholesterol is an established clinical indicator for the diagnosis of the MetS in the general population.\textsuperscript{21,23,29} Among women with PCOS, low HDL was confirmed in many studies and shown to be one of the most frequent among single abnormalities in MetS.\textsuperscript{9,15,16,25,28,37} Our results confirmed an abnormal HDL concentration within a selected cohort of Europid women with PCOS and MetS. Furthermore, HDL was shown to be an accurate marker, and together with LAP, the one most powerfully associated with MetS using either NCEP-ATP III, IDF or JIS criteria. Recently it was suggested that HDL may induce nitric oxide dependent vasorelaxation and consequent cardiovascular protection.\textsuperscript{38} Therefore, it was hypothesized that low HDL might be considered the most important factor associating PCOS with a disposition to cardiovascular diseases.\textsuperscript{9}

Insulin resistance is a prevalent metabolic condition in the vast majority of subjects with multiple metabolic disorders and can be easily estimated using HOMA-IR index.\textsuperscript{39} Via this simple method, the degree of insulin resistance is correlated with other metabolic abnormalities.\textsuperscript{40} It was shown that women with PCOS are at increased risk for the spectrum of disorders associated with insulin resistance, including MetS.\textsuperscript{41} Although direct measure of insulin resistance is not included in any of the criteria for the MetS used in this study, the cut-off value of HOMA-IR appeared to attain a significant level of diagnostic accuracy for the presence of MetS by all three international criteria used. Even more, it was shown that the assessment of insulin resistance, given as basal insulin concentrations or calculation of HOMA-IR, had a significant positive correlation with LAP in PCOS women who had MetS. Our results are in accordance with a study by Wiltgen et al\textsuperscript{33} showing an association of LAP with HOMA-IR. However, this study was performed on a smaller group of predominantly overweight/obese Caucasian PCOS women.

A limitation of our study is the relatively small control group for comparison with the whole group of PCOS. However, this difference was reduced or non-existent when the analysis of PCOS women was performed in relation to the presence of MetS. Another limitation could be the relatively younger
age of the PCOS women in relation to the controls. This limitation was also considered in our recent study,10 and hence all the analyses between groups were undertaken with the age adjustment. We are also aware that our PCOS women were enrolled from a population referred to the health care center and not from the general population, therefore, our results cannot be extrapolated to the general population. The method used for the measurement of androgens in the blood could be another limitation as we did not measure free testosterone but rather calculated FAI. Direct measurement of free testosterone concentration is related to technical difficulties with different assays that are not easily feasible.42 However, calculated FAI was proven to be of high diagnostic accuracy for diagnosing hyperandrogenemia.43

In conclusion, LAP is shown to be a simple and independent clinical indicator for the assessment of MetS in PCOS women of Caucasian origin. The high diagnostic accuracy of LAP that implies determination of WC and serum triglycerides is superseding the need for the use of multiple clinical indicators for the assessment of lipid accumulation as a prerequisite for diagnosis of metabolic syndrome and consequent prediction of cardiovascular diseases in PCOS women.

GRANT

This work was supported by Grants 41009 and 175032 from the Serbian Ministry of Science and Education.

DISCLOSURE SUMMARY

The authors have nothing to disclose.

REFERENCES

11. Coviello AD, Legro RS, Dunai A, 2006 Adolescent girls with polycystic ovary syndrome have an increased risk of the metabolic syndrome associated with increasing androgen levels independent of obesity and insulin resistance. J Clin Endocrinol Metab 91: 492-497.
17. Rotterdam ESHRE/ASRM-Sponsored PCOS consensus


Long-term follow-up results of growth hormone therapy for patients with adult growth hormone deficiency

Hidetoshi Ikeda,1 Masataka Kudo1,2

1Research Institute for Pituitary Disease, Southern Tohoku General Hospital, Koriyama, Japan, 2Division of Nephrology, Endocrinology, and Vascular Medicine, Tohoku University Graduate School of Medicine, Aoba-ku, Sendai, Japan

ABSTRACT

OBJECTIVE: We evaluated the long-term effects of growth hormone (GH) on markers of quality of life, glucose metabolism, and lipid metabolism to validate the adequacy of long-term GH replacement therapy for adult GH deficiency (AGHD). DESIGN: Eighty-three of 100 sequentially followed patients who received GH therapy were selected for this study. Forty-nine were men aged 26 to 78 years (mean, 52 years) and 34 were women aged 20 to 78 years (mean, 56 years). The GH-releasing peptide-2 stimulation test and arginine stimulation test were used to diagnose AGHD. The adult hypopituitarism questionnaire (AHQ) and biochemical parameters such as cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol, and glyated hemoglobin (HbA1c) were determined before treatment, at 6 months of treatment, and at 1, 2, 3, 4, 5, 6, 7, and 8 years of treatment. Considering age and sex as factors potentially influencing the effect of GH therapy, the patients were divided into age groups of <60 and ≥60 years and sex groups of men and women. Repeated measured analysis of variance (ANOVA) was employed. RESULTS: ANOVA demonstrated significant changes in mean AHQ scores during follow-up. Comparison of individual AHQ scores with baseline values revealed sequential improvements, stabilization, and decline in QOL. A significant elevation in HbA1c level was demonstrated. LDL-C and HDL-C levels changed significantly upon GH treatment regardless of sex or age. Levels of glucose, TC or TG did not change significantly. CONCLUSION: The effect of GH therapy on QOL showed sequential improvements and stabilization until 6-year follow-up.

Key words: Growth hormone deficiency, Growth hormone therapy, Long-term follow-up, Quality of life, Pituitary

INTRODUCTION

Many of the functions of growth hormone (GH) in humans are not known. It is involved in the regulation of body fluids, metabolism of sugars and fats, and possibly heart function. GH promotes lipid use by promoting triglyceride (TG) breakdown and
oxidation in cells. It is involved in the regulation of blood glucose. GH suppresses the ability of insulin to promote glucose uptake in peripheral tissues. Benefits of GH supplementation for the treatment of GH deficiency include reduced fat mass, increased lean mass, increased bone density, improved lipid profile, and reduction in the risk of cardiovascular disease. These risk factors are favorably influenced by GH replacement therapy.

Adults with GH deficiency complain frequently of low energy levels, emotional lability, and mental fatigue that result in a low perceived quality of life (QOL). Adults with GH deficiency often have a higher prevalence of depression than adults not suffering from GH deficiency. GH replacement has been proposed as a treatment for depression as a result of GH deficiency, but the long-term effects of such therapy are not known. Hypomyelination in a mouse model of GH deficiency has been found to be due to arrested proliferation of glial cells and to be reversed by GH replacement.

Thus, the GH-insulin-like growth factor 1 (IGF-1) axis is involved in regulation of the growth, development, and myelination of the brain. In addition, GH and IGF-1 affect cognition and biochemistry in the adult brain. Accordingly, accumulating evidence suggests that GH is closely associated with neuroprotection, regeneration, and functional plasticity in the adult brain.

However, concerns over the effectiveness of GH treatment in adults have been raised in the context of short-term follow-up. Only a few reports have addressed the beneficial effects of long-term GH treatment for adult GH deficiency (AGHD), particularly with regard to patient age and sex. Claessen et al reported the metabolic profiles after a minimum of 10 years of GH treatment in 98 patients with AGHD. They found that the prevalence of metabolic syndrome increased significantly during GH treatment, although an improvement in several cardiovascular risk factors was observed. Bunderen et al studied how GH treatment influences mortality and found that the mortality rate of patients with AGHD treated with GH supplementation did not differ from the control population regardless of sex. On the other hand, Gaillard et al reported a modest increase in mortality in patients with hypopituitarism who received GH replacement.

We wished to evaluate the long-term QOL for GH treatment of AGHD in addition to carrying out measurement of biochemical markers because these factors may influence mortality and development of the metabolic syndrome.

**MATERIALS AND METHODS**

The effectiveness of GH treatment is recognized in Japan and GH therapy was approved by the National Health Insurance Policy in 2006. In October 2009, hypopituitarism was included in the Research Project on Overcoming Intractable Diseases, which allows affected patients to receive financial assistance from the Department of Health and Welfare. Since then, this therapy has become much more readily available. Under these circumstances, 100 of 2000 patients who underwent pituitary surgery by one of the authors (H.I.) received GH therapy for 8 years.

Data were missing for 6 patients due to aggravation of diabetes mellitus, 6 patients who were victims of the tsunami in northern Japan in 2011, 2 patients with recurrence of brain tumors, and 2 patients who had moved to facilities in which self-injection was difficult. One patient was lost to follow-up.

Except for these 17 patients, the remaining 83 patients (94%) continued GH treatment irrespective of its efficacy. However, data for 12 out of the 83 patients were not measured at some time points, consequently statistical analyses were not carried out in these patients. Therefore, physical-examination and complete follow-up data were available for 71 patients. Five patients were followed up for 8 years, 6 patients for 7 years, 8 patients for 6 years, 23 patients for 5 years, 42 patients for 4 years, 45 patients for 3 years, 51 patients for 2 years, 64 patients for 1 year, and 71 patients for 6 months. We continued GH replacement therapy irrespective of whether GH administration was efficacious or not. As a result, data were missing for only 6% of participants and there was thus little risk of selection bias. Forty-nine patients were men aged 26 to 78 years (mean, 52 years) and 34 were women aged 20 to 78 years (mean, 56 years). Fifty-five patients had pituitary adenomas, 13 had craniopharyngiomas, 13 had Rathke’s cleft cysts, 1 had a meningioma, and 1 had hypophysitis. The backgrounds of the patients are summarized in Table 1.
The research protocol was approved by our institutional review board. The study was approved by the appropriate ethics committee and performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All patients provided written informed consent.

DEFINITION OF GROWTH HORMONE DEFICIENCY

Prior to treatment of pituitary diseases, evaluation for the presence of growth hormone deficiency (GHD) must be performed in all patients in whom surgery is indicated. A low reserve of GH secretion is detected using GH secretory stimulation tests, namely, the insulin tolerance test, arginine tolerance test, L-dopa tolerance test, glucagon tolerance test, and GH-releasing peptide-2 (GHRP-2) tolerance test. In this study, the GHRP-2 tolerance test was performed first, followed by the arginine tolerance test if a second test was deemed necessary. For the arginine tolerance test, 30 g of arginine hydrochloride in 300 mL of normal saline were infused intravenously over 30 minutes and blood samples were collected before and at 60, 90, and 120 minutes after infusion for determination of serum GH levels. For the GHRP-2 stimulation test, blood samples were collected before and 15, 30, 45, and 60 minutes after intravenous injection of 100 µg of pralmorelin hydrochloride. AGHD was defined as a peak GH level of <1.8 ng/mL for the arginine test and <9 ng/mL for the GHRP-2 stimulation test.

QOL measurement

Symptoms of GHD were considered to be fatigue, a low energy level, low concentration levels, decreased vigor, depression, and low sexual desire. Signs associated with AGHD were considered to be dry and thinning skin, softened body hair, increased body fat (fat on the internal organs), an increased waist/hip ratio, a decrease in lean body weight, and decreased bone mass and muscular strength. Generic or disease-specific QOL scales can be used to measure QOL in patients with hypopituitarism: Nottingham Health Profile; Psychological General Well Being Index; MOS 36 Item Short Form Health Survey (SF-36); Quality of Life-Assessment of Growth Hormone Deficiency in Adults (QoL-AGHDA); Questions on Life Satisfaction (QLS).

The adult hypopituitarism questionnaire (AHQ) was developed based on two domains: psychosocial and physical. The psychosocial domain was divided into six subdomains. The physical domain was divided into seven subdomains. Cronbach’s alpha coefficient was 0.72-0.93 for the psychosocial subdomain and 0.73-0.89 for the physical subdomain, denoting acceptable internal consistency. With regard to reproducibility, the intraclass correlation coefficient was 0.77-0.90 for psychosocial subdomains and 0.86-0.94 for physical subdomains, and reproducibility was considered sufficient. AHQ subdomains correlated moderately with all of SF-36v2 subdomains (0.13-0.68). Since the AHQ addresses all of these signs and symptoms of

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of pts.</th>
<th>Sex</th>
<th>Age group</th>
<th>Age at diagnosis of GHD (y.o.)</th>
<th>Height (cm)</th>
<th>Body weight (kg)</th>
<th>BMI (%)</th>
<th>Periods between diagnosis and GH introduction (months)</th>
<th>Thyroid suppl.</th>
<th>Steroid suppl.</th>
<th>Testosterone suppl.</th>
<th>Anti-hyperlipidemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>34</td>
<td>M</td>
<td>&lt;60</td>
<td>47.6±8.4</td>
<td>165.9±7.6</td>
<td>71.7±21.7</td>
<td>25±7.2</td>
<td>5.3±4.3</td>
<td>60%</td>
<td>70%</td>
<td>30%</td>
<td>30%</td>
</tr>
<tr>
<td>B</td>
<td>14</td>
<td>M</td>
<td>≥60</td>
<td>66±4.9</td>
<td>164.9±7.4</td>
<td>70.3±12.9</td>
<td>25.9±4.5</td>
<td>7.9±5.1</td>
<td>79%</td>
<td>71%</td>
<td>36%</td>
<td>36%</td>
</tr>
<tr>
<td>C</td>
<td>19</td>
<td>F</td>
<td>&lt;60</td>
<td>47.3±11.3</td>
<td>156.4±5.1</td>
<td>62.1±8.9</td>
<td>25.4±3.3</td>
<td>8.6±13.1</td>
<td>89%</td>
<td>79%</td>
<td>0%</td>
<td>78%</td>
</tr>
<tr>
<td>D</td>
<td>16</td>
<td>F</td>
<td>≥60</td>
<td>67.2±5.8</td>
<td>153.4±5.8</td>
<td>55.5±11.5</td>
<td>23.6±4.6</td>
<td>7.9±7.5</td>
<td>81%</td>
<td>75%</td>
<td>0%</td>
<td>63%</td>
</tr>
</tbody>
</table>

Results are presented as mean ± SD.

A group: Male, under 60 years old; B group: Male, more than 60 years old; C group: Female, under 60 years old; D group: Female, more than 60 years old.

BMI: body mass index; GH: growth hormone; GHD: growth hormone deficiency; pts: patients; Suppl.: supplementation; y.o.: years old.
AGHD and has been shown to be suitable for Japanese patients, this questionnaire was used for evaluation. The same questionnaire was repeatedly used before treatment, at 6 months of treatment, and at 1, 2, 3, 4, 5, 6, 7, and 8 years of treatment.

**Biochemical parameters**

To clarify the effect of long-term GH replacement on the metabolism of lipids and sugar and cardiovascular risk factors, we selected biochemical markers: fasting serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL)-cholesterol, low-density lipoprotein (LDL)-cholesterol, glucose, and glycated hemoglobin (HbA1c). These biochemical parameters were measured before treatment, at 6 months of treatment, and at 1, 2, 3, 4, 5, 6, and 7 years of treatment; the levels of these values were assessed to determine whether GH therapy had significant effects on lipid and glucose metabolism. The sequential observation period for each parameter lasted from 6 months to 7 years (mean, 5 years).

**Method of GH administration**

GH was administered daily as an intradermal injection before sleep at an initial dose of 3 µg/kg body weight. Unless there were clear adverse effects, the dose was increased every 4 weeks with the goal of maintaining insulin-like growth factor 1 (IGF-1) levels within the expected ranges for age and sex.

**Comparison of groups by age and sex**

Most patients were in their fifties, followed by patients in their sixties; the mean age of men was 52 years and that of women was 56 years. In light of these findings, it seemed appropriate to divide the patients into two age groups with a cutoff of 60 years. We compared the effect of GH treatment between the following groups of patients: men and women aged <60 years and men and women aged >60 years.

**Statistical analysis**

For comparisons within groups, repeated measures analyses (ANOVA) using StatView v4.0 was used for continuous data to compare differences in parameters before and after GH treatment. If significant differences were detected, Wilcoxon signed rank analyses were carried out. The difference in QOL score from baseline to each post-baseline period (6 months to 8 years) at each time point was tested using Wilcoxon signed rank analyses. The Kruskal-Wallis test was used to evaluate the differences in background factors (Table 1) among the four groups divided by age and sex. Welch’s t-test was used to compare the differences of two independent groups. A p value of <0.05 was taken to be statistically significant.

**RESULTS**

Table 1 shows a summary of the demographic and clinical characteristics of the patients. The Kruskal-Wallis test revealed no significant difference between the two groups in background factors such as body mass index (BMI) and period of time between the diagnosis of GHD and introduction of GH therapy. Age at diagnosis of GDH was not significantly different between men and women (p=0.18).

No significant differences in either weight or height were observed between the two male age groups or between the two female age groups. Considering the serum IGF-1 SD scores, the dose of GH administered was ideal until 6 months after GH supplementation. Sequential changes in the hormone supplementation dose were observed only for thyroxin supplementation. The daily dose of thyroxin had to be increased after 6 months of GH supplementation therapy in 8 of the 18 men aged <60 years (44%), 6 of the 11 men aged <60 years (55%), 5 of the 17 women aged <60 years (29%), and 8 of the 13 women aged <60 years (62%).

Sequential changes in the IGF-1 concentration and IGF-1 SD score among the four groups are shown in Table 2. Both the IGF-1 concentration and IGF-1 SD score were significantly higher than the pretreatment values and remained high in each group (Table 2).

**Evaluation of the AHQ**

Sequential changes in QOL and biochemical parameters upon GH supplementation varied widely among patients. Psychosocial AHQ scores changed significantly upon GH treatment at 6 months as well as 1-, 2-, 3-, 4-, 5-, 6-, 7-, and 8-year time points by ANOVA (Figure 1). A significant difference in the psychosocial domain between males and females at 6- and 7-year time points was noted (Figure 2). ANOVA also demonstrated significant changes in mean values of physical AHQ scores at 6 months as well as 1-, 2-, 3-, 4-, 5-, 6-, and 7-year time points.
Table 2. a) Sequential changes of serum IGF-1 value after GH treatment

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age</th>
<th>Pre GH treatment</th>
<th>6M</th>
<th>1Y</th>
<th>2Y</th>
<th>3Y</th>
<th>4Y</th>
<th>5Y</th>
<th>6Y</th>
<th>7Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>&gt;60Y</td>
<td>94.8±44.7</td>
<td>176±61.1*</td>
<td>189±88.8*</td>
<td>178±43.1*</td>
<td>161±32.4*</td>
<td>158±29.6*</td>
<td>161±22.8*</td>
<td>162±22.6*</td>
<td>174±19.1</td>
</tr>
<tr>
<td></td>
<td>N=29</td>
<td></td>
<td>(N=29)</td>
<td>(N=25)</td>
<td>(N=18)</td>
<td>(N=15)</td>
<td>(N=11)</td>
<td>(N=6)</td>
<td>(N=4)</td>
<td>(N=2)</td>
</tr>
<tr>
<td></td>
<td>≤60Y</td>
<td>85±48.2</td>
<td>157±39.7*</td>
<td>148±46.8*</td>
<td>139±41.3*</td>
<td>130±35.5*</td>
<td>121±33.2*</td>
<td>139±28.7</td>
<td>119±16.2</td>
<td>156±19.1</td>
</tr>
<tr>
<td></td>
<td>N=20</td>
<td></td>
<td>(N=20)</td>
<td>(N=19)</td>
<td>(N=17)</td>
<td>(N=15)</td>
<td>(N=9)</td>
<td>(N=5)</td>
<td>(N=3)</td>
<td>(N=2)</td>
</tr>
<tr>
<td>Female</td>
<td>&gt;60Y</td>
<td>91±45.9</td>
<td>161±50.3*</td>
<td>156±64.7*</td>
<td>148±53.5*</td>
<td>127±42.6*</td>
<td>134±41.9</td>
<td>150±30.9</td>
<td>153±32.5</td>
<td>N=1</td>
</tr>
<tr>
<td></td>
<td>N=14</td>
<td></td>
<td>(N=12)</td>
<td>(N=12)</td>
<td>(N=9)</td>
<td>(N=7)</td>
<td>(N=3)</td>
<td>(N=2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>≤60Y</td>
<td>86.8±60.7</td>
<td>149±64.5*</td>
<td>156±44.5*</td>
<td>161±59*</td>
<td>154±26.8*</td>
<td>163±40.5*</td>
<td>167±21.9</td>
<td>185±26.3</td>
<td>N=1</td>
</tr>
<tr>
<td></td>
<td>N=21</td>
<td></td>
<td>(N=21)</td>
<td>(N=19)</td>
<td>(N=14)</td>
<td>(N=10)</td>
<td>(N=3)</td>
<td>(N=2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*: significant; (p<0.05)

Data are compared with pre GH treatment and 6M, 1Y, 2Y, 3Y, 4Y, 5Y, 6Y, 7Y after GH treatment.

IGF-1 value; Mean ±SD; N: number of patients

Table 2. b) Sequential changes of IGF-1 SD score after GH treatment

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age</th>
<th>Pre GH treatment</th>
<th>6M</th>
<th>1Y</th>
<th>2Y</th>
<th>3Y</th>
<th>4Y</th>
<th>5Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>&gt;60Y</td>
<td>-2.32±1.82</td>
<td>0.08±1.51*</td>
<td>0.3±1.71*</td>
<td>0.48±1.07*</td>
<td>0.2±0.79*</td>
<td>0.18±0.69*</td>
<td>0.25±0.63*</td>
</tr>
<tr>
<td></td>
<td>N=29</td>
<td></td>
<td>(N=29)</td>
<td>(N=25)</td>
<td>(N=18)</td>
<td>(N=15)</td>
<td>(N=11)</td>
<td>(N=6)</td>
</tr>
<tr>
<td></td>
<td>≤60Y</td>
<td>-1.84±1.81</td>
<td>0.56±1.02*</td>
<td>0.27±1.1*</td>
<td>0.11±1.14*</td>
<td>-0.11±0.92*</td>
<td>-0.33±1.01*</td>
<td>0.28±0.56</td>
</tr>
<tr>
<td></td>
<td>N=20</td>
<td></td>
<td>(N=20)</td>
<td>(N=19)</td>
<td>(N=17)</td>
<td>(N=15)</td>
<td>(N=9)</td>
<td>(N=5)</td>
</tr>
<tr>
<td>Female</td>
<td>&gt;60Y</td>
<td>-2.43±1.95</td>
<td>0.14±0.86*</td>
<td>-0.33±2.37*</td>
<td>-0.38±1.15*</td>
<td>-1±1.12*</td>
<td>-0.5±1.23*</td>
<td>-0.5±0.56</td>
</tr>
<tr>
<td></td>
<td>N=13</td>
<td></td>
<td>(N=13)</td>
<td>(N=12)</td>
<td>(N=9)</td>
<td>(N=7)</td>
<td>(N=3)</td>
<td>(N=3)</td>
</tr>
<tr>
<td></td>
<td>≤60Y</td>
<td>-1.5±2.29</td>
<td>0.74±1.75*</td>
<td>1.19±1.14*</td>
<td>1.24±1.27*</td>
<td>1.16±0.61*</td>
<td>1.35±0.89*</td>
<td>1.37±0.25</td>
</tr>
<tr>
<td></td>
<td>N=21</td>
<td></td>
<td>(N=21)</td>
<td>(N=19)</td>
<td>(N=14)</td>
<td>(N=12)</td>
<td>(N=10)</td>
<td>(N=3)</td>
</tr>
</tbody>
</table>

*: significant (p<0.05);

Data are compared with pre GH treatment and 6M, 1Y, 2Y, 3Y, 4Y, 5Y, 6Y, 7Y after GH treatment.

IGF-1SD score; Mean ±SD; N: number of patients

---

**Figure 1.** Sequential changes in mean AHQ (psychosocial) scores upon GH treatment.

**Figure 2.** Sequential changes in mean AHQ (psychosocial) scores upon GH treatment in males and females.
However, there were no significant differences in the physical AHQ domain with respect to age and sex.

Comparison of individual AHQ values with baseline values revealed sequential improvements, stabilization, and decline in QOL. Sequential changes in mean psychosocial AHQ values revealed a decline at 7-year follow-up (Figure 1). Physical AHQ values also revealed a decline at 6-year follow-up (Figure 3). In this context, mean values of psychosocial and physical scores tended to become lower than baseline values at 7-8-year follow-up.

**Evaluation of metabolic markers**

ANOVA demonstrated significant changes in mean values of HbA1C at 1, 2, 3, 4, and 5 years (Figure 4). Comparison of baseline values of HbA1C with sequentially measured mean values of HbA1C showed that HbA1c levels increased significantly from 6-month to 6-year follow-up (Table 3). ANOVA demonstrated significant changes in mean values of HDL-C at 1, 2, 3, 4, and 5 years (Figure 5). Comparison of baseline values of HDL-C with sequentially measured mean values of HDL-C revealed that HDL-C levels increased significantly from 4-year to 6-year follow-up (Table 3).

**Table 3. Effect of GH replacement on metabolic markers**

<table>
<thead>
<tr>
<th>Description</th>
<th>Baseline</th>
<th>Month 6</th>
<th>Year 1</th>
<th>Year 2</th>
<th>Year 3</th>
<th>Year 4</th>
<th>Year 5</th>
<th>Year 6</th>
<th>Year 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA1C</td>
<td>5.626 ± 0.610</td>
<td>5.815 ± 0.678 *</td>
<td>5.88 ± 0.566 *</td>
<td>5.888 ± 0.515 *</td>
<td>5.984 ± 0.558 *</td>
<td>6.067 ± 0.596 *</td>
<td>6.108 ± 0.749 *</td>
<td>6.1 ± 0.954</td>
<td></td>
</tr>
<tr>
<td>TG</td>
<td>151.671 ± 71.765</td>
<td>181.605 ± 137.976</td>
<td>162.397 ± 94.528</td>
<td>160.75 ± 67.721</td>
<td>148.18 ± 58.016</td>
<td>163.2 ± 109.393</td>
<td>163.75 ± 4.006</td>
<td>152 ± 77.286</td>
<td>127 ± 54.148</td>
</tr>
</tbody>
</table>

All blood samples were taken after fasting.
Data are presented as the mean ± SD. *: p<0.05; compared with baseline.
Significant changes in mean values of LDL-C were observed at 3, 4, 5, and 6 years (Figure 6). Comparison of baseline values of LDL-C with sequentially measured mean values of LDL-C revealed that LDL-C levels decreased significantly from 1-year to 4-year follow-up (Table 3). There were no significant changes in mean values of TG, TC or glucose during follow-up. Changes in serum glucose were not related to changes in the duration of GH treatment at any time point (3 years, p=0.29; 4 years, p=0.43; 5 years, p=0.79; 6 years, p=0.69). Changes in serum TG were not related to changes in the duration of GH treatment at any time point (3 years, p=0.36; 4 years, p=0.50; 5 years, p=0.62; 6 years, p=0.32). Changes in TC were not related to changes in the duration of GH treatment except at 4 years (1 year, p=0.88; 2 years, p=0.58; 3 years, p=0.21; 4 years, p=0.04; 5 years, p=0.42; 6 years, p=0.36). The age and sex of patients had no significant effect on their levels of metabolic markers.

**DISCUSSION**

AGHD adversely affects patients’ cardiovascular risk profile, bone density, and QOL. GH replacement, when dose-titrated, is well tolerated and has been shown to have beneficial effects on patients’ body composition, lipid profile, bone mineral density, and psychological well-being. Patients with AGHD on 5-year GH replacement appear to demonstrate mortality rates similar to those of country-specific background populations, in contrast to a previous report.

Patients with AGHD have also been demonstrated to have lower QOL than control individuals. The Nottingham Health Profile, Psychological General Well-Being Index, MOS 36-Item SF-36, QoL-AGHDA, and QLS-hypopituitarism (QLS-H) have been used in European countries and the United States to evaluate QOL. Because the NHP and PGWB Index are not disease-specific but are instead used for many types of diseases without focusing on specific characteristic problems occurring in patients with GHD, these questionnaires lack sensitivity in evaluation of such patients. Although previous reports have indicated that QOL improvement by GH administration can be evaluated by the QoL-AGHDA or OLS-H in patients from both Europe and the United States, these tools are not suitable for QOL evaluation in Japanese patients with AGHD because of the absence of a large difference between GH-treated and placebo-treated control groups. Thus, the Japanese AHQ was developed to assess QOL parameters for Japanese patients with hypopituitarism.

When the effects of GH administration data were studied, QOL appeared to improve during long-term GH replacement, although the majority of the beneficial effects on QOL were typically seen within the first year of treatment. Svenson et al and Holdaway et al, using a follow-up of 3 years, reported improvement in QOL. Wiren et al, using a follow-up of 20-50 months, reported that the QOL score improved significantly compared with the baseline score. Jorgensen et al reported that QOL improvement was significant in the early years for most subgroups but that some of the observed improvement 6-10 years later did not reach significance (which may have been because of missing data). The reason why some results did not reach significance is because of the small sample size in later years and because in that observational study data were gathered from several countries. A limitation of this type of observational study is that patients unresponsive to GH therapy are lost to the study spontaneously.

In our study, ANOVA demonstrated significant changes in mean values of AHQ scores (psychosocial domain) at 6 months as well as at 1, 2, 3, 4, 5, 6, 7, and 8 years. A significant difference in the psychosocial domain was noted between males and females at 6 and 7 years. ANOVA also demonstrated
significant changes in mean values of AHQ scores (physical domain) at 6 months as well as at 1, 2, 3, 4, 5, 6, and 7 years. Sequential changes in mean AHQ (psychosocial) values declined at 7-year follow-up and the AHQ (physical) value also declined at 6-year follow up. Mean values of psychosocial and physical scores became lower than baseline values at 7-8-year follow-up, a finding that is consistent with the report of Jorgensen et al.16

Comparison of individual AHQ scores with baseline values showed sequential improvements, stabilization, and decline in QOL. Ten-year follow-up data gathered from Europe and the USA by Mo et al17 revealed that QOL improvements remained significant throughout that decade. In that study17 various physicians attended to patients, which may have led to biases in study results (e.g., selection bias, unresponsive patients lost to the study). Owing to these selection biases, the trend of decline in QOL may have disappeared from their data. Conversely, our study was carried out by only one physician, which resulted in identical treatment and follow-up for all patients. In addition, we continued GH replacement irrespective of whether GH administration was efficacious or not. Data were missing for only 6% of our study cohort, thus there was little scope for selection bias. Hence, our study design was ideal for evaluation of the true efficacy of GH replacement compared with the large cooperative study by Mo et al.17 The score range of the psychosocial domain was 7 to 167 (range: 0-204) and that of the physical domain was 9 to 181 score (range: 0-252), so there were no peaks or troughs in values.

During long-term follow-up, some patients suffered from other disorders (e.g., femoral fracture, esophageal cancer, hemorrhagic colon, multiple diverticula), which led to a reduction in QOL. Moreover, patients who continued GH replacement for >5 years had a decline in QOL because of aging. These two effects could also have been responsible for the decline in QOL. QOL is reportedly strongly influenced by BMI; however, the BMI in our four groups of patients did not differ significantly according to the Kruskal-Wallis test.

The purpose of GH therapy is not only to improve QOL after it has been impaired by GH deficiency, but also to correct metabolic abnormalities such as increased body fat, abnormal body composition including a decrease in lean body weight, and abnormal blood lipid levels. Low-dose GH supplements therapy for 6 months in patients with AGHD significantly improved both the total and LDL-cholesterol levels compared with the pretreatment levels. Such improvements reportedly continue for 2 years on GH treatment.6 Therefore, we measured the fasting total cholesterol, triglyceride, HDL-cholesterol, and LDL-cholesterol levels before and sequentially during treatment.

There are controversial publications concerning the effect of GH supplementation on various factors such as QOL, mortality, lipid metabolism, and glucose metabolism. There are conflicting reports from Europe and the United States regarding diabetic patients. One study recommended not to treat diabetic patients with GH and another concluded that GH treatment posed no risk of increasing either the glucose or HbA1c level.18,19 In our study, significant changes in mean values of HbA1C were demonstrated at 1, 2, 3, 4, and 5 years (ANOVA) and there were no significant changes in glucose levels during long-term GH treatment.

Holdaway et al,14 using a follow-up of 3 years, reported no significant changes in levels of TC, LDL-C, TG, fasting blood sugar or HbA1C. Jorgensen et al,16 using a follow-up of 42 months, reported significant changes in levels of LDL-C and HDL-C, but not in levels of TG, HbA1C, TC or glucose. Our study demonstrated significant changes in mean levels of LDL-C at 1, 2, 3, 4, and 5 years (ANOVA), a result that is in accordance with that of Jorgensen et al.16 Significant changes in mean levels of LDL-C were noted at 3, 4, 5, and 6 years (ANOVA), a result that is in accordance with that of Jorgensen et al.16 There were no significant changes during follow-up in levels of TG or TC, a result that is in accordance with that of Holdaway et al.14

For appropriate evaluation of GH therapy, further studies involving more subjects who are evaluated for an extended period of time are necessary.

CONCLUSION

In patients with AGHD, long-term GH administration showed sequential improvements, stabilization, and decline in QOL. GH therapy elicited significant
changes in the psychosocial domain of AHQ until 8-year follow-up. GH therapy elicited significant changes in the physical domain of AHQ until 7-year follow-up. Levels of biochemical markers such as HbA1C, HDL-C, and LDL-C showed significant changes during long-term follow-up.

REFERENCES

Corticotropin-releasing factor (CRF) system localization in human fetal heart

Efterpi Chouridou,1 Maria Lambropoulou,2 Maria Koureta,1 Christina Zarouchlioti,1 Ioanna Balgouranidou,1 Evangelia Nena,3 Nikolaos Papadopoulos,2 Ekaterini Chatzaki1

1Laboratory of Pharmacology, 2Laboratory of Histology-Embryology, 3Laboratory of Hygiene and Environmental Protection, Faculty of Medicine, Democritus University of Thrace, Dragana, Alexandroupolis, Greece

ABSTRACT

OBJECTIVE: The corticotropin-releasing factor (CRF) family consists of the neuropeptides CRF, Ucn I, II and III and the binding sites CRF1, CRF2 and CRF-BP. It regulates stress response and the homeostasis of an organism. In this study, we examined the presence of the CRF system in the human hearts of normal and pathological fetuses. DESIGN: Heart tissues from 40 archival human fetuses were divided into Group A (without pathology, ‘normal’), Group B (with chromosomal abnormalities) and Group C (with congenital disorders). Immunohistochemistry was used to localize the CRF system. Results correlated to gestational trimester and pathology. RESULTS: Immunoreactivity for all antigens was found in cardiac myocytes of all groups, in almost all samples, except Ucn III which was present in almost half of the fetuses of Groups B and C and was not detected at all in Group A. Ucn III was more often present during the earlier stage of development (<21weeks) and in fetuses with congenital disorders. In a fetus diagnosed with heart pathology, all but Ucn III antigens were also present. CONCLUSIONS: We localized a complete CRF system in the human fetal heart and correlated the presence of Ucn III to development and pathology. More studies are needed to verify and clarify the exact role of the CRF system in the human fetal heart.

Key words: CRF, CRF receptors, Development, Fetal, Heart, Human, Urocortins

INTRODUCTION

Harris was the first to suggest that the hypothalamus regulates the secretion of the adrenocorticotropic hormone (ACTH) via neurochemical factors.1 His theory was documented experimentally in the fifties by Saffran2 and was proven by Vale et al much later3 when a 41 amino acid peptide was isolated from the ovine hypothalamus. This peptide was originally named corticotropin-releasing hormone (CRH) and later corticotropin-releasing factor (CRF) by R. Hauger,4 since the functions of the specific peptide were beyond those of a simple hormone. Today we refer to the CRF system or family, which consists of the endogenous neuropeptides CRF, Urocortin I (Ucn I, 40 aa peptide), Urocortin II (Ucn II or stresscopin-
related peptide, 38 aa) and Urocortin III (Ucn III or stresscopin, 38 aa), the receptors CRFR1 (415-446 aa) and CRFR2 (both belonging to the class B/secretin family of G-protein coupled receptors and both presenting various isoforms) and the CRF-binding protein (CRF-BP, a glycoprotein of 37-kDa with 322 aa). All neuropeptides bind with CRFR2, while Ucn II and III have moderate or no correlation with CRF-BP and CRFR1.5-8

In general, the CRF system is responsible for homeostasis9 and stress response regulation at multiple levels (neuronal, endocrine and immunological), while it is additionally connected to the phenomena of inflammation10 and apoptosis.11-15 CRF system members are detected at multiple sites of the central nervous system (CNS) and in the periphery. A significant number of published studies have documented an important role of the CRF system within the cardiovascular system.16-24 In the adult heart, CRF and/or its ligands have been found to be expressed in both animals and humans. More specifically, they were identified in the heart of rodents,25 including rats26,18 and mice,21 dogs27 and Tupaia belangeri.28 In adult humans on the other hand, an immunohistochemical study revealed Ucn I immunoreactivity in myocytes of the normal heart, which was more intense in the diseased heart, indicating, together with evidence of its positive inotropic action, its possible role in the pathophysiology of cardiac hypertrophy or the failing heart.11 In addition, Ucn II transcripts were detected at high levels of expression in human heart tissues.6 According to another study, Ucn I is produced in the human heart, where it is stored, and can exert its effects via CRF-R2 in an autocrine and/or paracrine way.29

Interestingly, Baigent supported the view that since the adult heart secretes Ucn I, most probably the respective fetal tissue should also secrete the same neuropeptide.30 However, so far there has been no confirmation of the presence of the CRF family in the fetal heart of animals or humans. In the human, limited information is offered regarding the presence of the CRF system in fetal tissues and its contribution to fetal maturation and/or pathology. Recently we reported the presence of all CRF family members in human fetal lungs during development,31 while CRFR1 mRNA had previously been found in human fetal adrenals.32 In animal fetal tissues, studies have reported the presence of CRF family members centrally in the rat and ovine hypothalamus,33,34 in the ovine pituitary,35 hippocampal-amygdala complex, frontal cerebral cortex (FCC) and brainstem16 and in the mouse cerebellum.37 In the periphery, the CRF family was detected in the rat fetal pancreas and GI tract18 and in the fetal ovine39 and sheep colon.40

In the present study, we examined by immunohistochemistry the presence of CRF neuropeptides and their binding sites in the heart of human fetuses. Our samples were obtained following spontaneous abortions and curettages and are representative of different gestational ages and pathology, including congenital or chromosomal disorders. Of these, a case study of a fetus diagnosed with heart pathology is presented separately.

MATERIALS AND METHODS

Tissues

Fetal heart tissues were retrieved from 40 archival human fetuses in the Histology-Embryology Laboratory Tissue Bank of the Democritus University of Thrace (DUTH), Alexandroupolis, Greece. All standard pathological examination and diagnosis data concerning the fetuses were available, as well as all relevant medical information on the mothers. All fetuses were derived from spontaneous abortions and curettages due to medical reasons involving the mother (elective therapeutic termination of pregnancy). Fetuses with nuchal cord were excluded from our study. Fetuses with no congenital or chromosomal anomalies and no signs of chorioamnionitis were considered as ‘normal’ (Group A, total n=15, all male). Pathological fetuses were divided into two groups: Group B (total n=4, male:2, female: 2) included fetuses with chromosomal abnormalities (Down syndrome and Edward’s syndrome) and Group C (total n=21, male:13, female:8) with congenital malformations (of the Nervous System, heart/central vessels, lungs, skeleton, abdominal wall, visceral cranium and face). It is important to note that some of the fetuses suffered from more than one pathology, as can be seen in Table 1. Fetuses were further divided into gestational trimesters, according to their gestational age, which ranged from 12 to 39 weeks and was estimated by the mother’s last menstrual period (LMP). The first 12 weeks of
Table 1. Characteristics and grouping of fetuses used in the study (Some of the pathological fetuses suffered from more than one pathology). Gestational age was estimated by mother’s last menstrual period (LMP).

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
<th>Gestational trimester (n)</th>
<th>Sex</th>
<th>Pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>First</td>
<td>Second</td>
<td>Third</td>
</tr>
<tr>
<td>A</td>
<td>15</td>
<td>0</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>0</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>21</td>
<td>1</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>A+B+C</td>
<td>40</td>
<td>1</td>
<td>31</td>
<td>8</td>
</tr>
</tbody>
</table>

Group A: fetuses with no pathological findings were considered ‘normal’. Group B: pathological fetuses with chromosomal abnormalities. Down syndrome (n=3), Edward syndrome (n=1), acute non-specific chorioamnionitis (n=1), hydropic degeneration of chorionic villi (n=1). Group C: pathological fetuses with congenital disorders of visceral cranium/face (n=6), skeleton (n=3), heart/central vessels (n=1)*, lungs (n=2), nervous system (n=7) and abdominal wall (n=1). Two fetuses suffered from hydropic degeneration of chorionic villi, 8 from acute chorioamnionitis, 2 from acute placenta, 2 from recessive fetal development and 1 from oligohydramnios.

*Heart pathology: presence of interventricular foramen, right ventricle hypertrophy and aortic translocation astride the interventricular septum.

pregnancy were considered as the first trimester, the period between the 13th and 24th gestational week the second trimester and beyond the 25th gestational week the third trimester. Following the classification of the fetuses, an autopsy was performed and heart tissues were obtained from the right or left fetal ventricle. The tissues were finally embedded in paraffin and sections were used for immunohistochemistry. The study protocol was approved by the Ethical Committee of the University Hospital of DUTH, (Decision no. 45/27/16-11-2009) and was conducted according to the guidelines for the analysis of fetal cells and tissues.

Antisera

The antisera used for CRF, CRFR2, Ucn I, II and III detection were obtained from Phoenix Pharmaceuticals (H-017-06, H-006-24, H-019-14, H-019-30, H-019-28, respectively; Belmont, Calif., U.S.A.). The antisera used for CRF was raised against the whole human peptide sequence: it is 100% specific for human, rat, mouse, canine and feline CRF and exhibits no cross-reactivity to other peptides. The specific antisera used for CRFR2 was raised against aa 385-411 of the human CRFR2 receptor. The specific antisera used for Ucn I was raised against the whole human Ucn I peptide sequence and is 100% specific for human and rat peptide. The specific antisera used for Ucn II was raised against aa 6-43 of the human Ucn II peptide sequence. The specific antisera used for Ucn III was raised against aa 3-40 of the human Ucn III peptide sequence. The CRF-BP antiserum was obtained from Santa-Cruz Biotechnology [CRF-BP (C-8): SC-365975]: it is a mouse monoclonal antibody specific for an epitope mapping between amino acids 299-322 at the C-terminus of CRF-BP of human origin. The anti-CRFR1 antiserum was the IgG-purified fraction of 4467a-CRFR1 which has previously been shown to be specific and selective for CRFR1. It was kindly donated by Dr. D. Grigoriadis, Neurocrine Bioscience Inc., San Diego, CA., U.S.A.

Immunohistochemistry

Immunohistochemistry was conducted as previously described. Tissue specimens were fixed in formalin and embedded in paraffin, according to standard procedures. Four-micron sections (4μm) of representative blocks were deparaffinized, rehydrated and treated with 0.3% H₂O₂ for 5 min in methanol to prevent endogenous peroxidase activity. After washing, slides were incubated at 4°C overnight with the primary rabbit anti-human polyclonal antiserum (anti-CRF 1:500, anti-Ucn I 1:500, anti-Ucn II 1:1000, anti-Ucn III 1:4000, anti-CRFR-BP 1:200, 4467a-CRFR1 1:7000, anti-CRFR2 1:1000, diluted in 10% normal rabbit serum in phosphate buffer saline, PBS). Control slides were incubated for the same period with normal rabbit serum IgG and were used as common negative control for all antibody staining. Immunostaining was detected by the Dako REAL TM EnVision TM Detection System, Peroxidase/DAB+, Rabbit/
Mouse kit (DAKO Denmark A/S, Denmark), using a standard streptavidin/biotin detection method, following the instructions of the manufacturer. Finally, bound antibody complexes were stained for 5 min with 0.05% diaminobenzidine, counterstained with Mayer’s haematoxylin, mounted and observed under a Nikon Eclipse 50i microscope. The same process was followed for all the negative control slides.

For each slide, approximately 10 fields of stained sections were evaluated by two independent observers and scored in a blinded fashion. Estimations by the two independent observers had an approximately 10% disagreement in most cases and was therefore considered insignificant. Every stained cell was scored as positive, regardless of its staining intensity. Positivity was graded in a four-scale system as follows: Grade 3 represents >70% positive cells in the total number of cells of the specific cell-type counted per field, Grade 2 between 40-70%, Grade 1 between 10-40% and Grade 0 stands for <10% positively stained cells. The extent of positive cells was assessed in cardiac myocytes.

**Statistical analysis**

Statistical significance was assessed by the chi-square test for categorical variables using SPSS 17.0 statistical software (SPSS Inc. Chicago, Illinois, USA). Significance was set at p value <0.05. Comparisons were made between positively stained (Grades 1, 2 and 3) and negative (Grade 0) tissues.

**RESULTS**

Cardiac myocytes were positively stained by immunohistochemistry for all antibodies, except Ucn III, which was not detected at all in Group A. Immunoreactivity was localized in the cell cytoplasm for all neuropeptides and CRF-BP, while for the receptors it was mainly membranic. Blood vessels and arteries were positive for all antibodies. Human placental tissue was also stained in parallel and was used as a positive control for all antigen staining (not shown). Representative pictures of fetuses of different gestational week, normal or pathological, are depicted in Figure 1. Accumulated results depicting fractions of positive tissues per study group are shown in Figure 2. Gestational age of fetuses was estimated by the mother’s last menstrual period (LMP).

**Immunohistochemical localization of CRF neuropeptides and receptors in the human fetal heart**

Results for CRF, Ucn I, II and III, receptors CRFR1, CRFR2 and CRF-BP immunohistochemistry with semi-quantitative evaluation in the human fetal heart are presented in Tables 2, 3 and 4 and Figure 2. Immunolocalization of CRF, Ucn I, Ucn II, CRFR1, CRFR2 and CRF-BP was found at different grades in all groups and gestational trimesters of our fetus groups. CRF staining was strongly positive (Grade 3) in all fetuses for all Groups and trimesters. Likewise, Ucn I presence was moderately to strongly positive (Grade 2-3) in all fetuses. Ucn II was also present in all but one tissue (female fetus, 20 w, with congenital disorders of visceral cranium/face and acute chorioamnionitis), in varying intensities (Grade 1-3, mostly 2). In contrast, Ucn III was weakly immunoreactive (Grade 1) in half of Group B fetuses and in a few (38.09%) of Group C, while it was absent in all fetuses of Group A and in fetuses of the third gestational trimester. In fact, the presence of Ucn III was significantly correlated to gestational age, as it was more frequently found before the 21st gestational week than in older fetuses (p=0.021), whereas statistical analysis between the 2nd and 3rd trimester fetuses showed marginally no significance (p=0.08). Presence of Ucn III was also correlated to the diagnosis of some kind of congenital disorder, as it was more often found in Group C than in Group A (normal fetuses) (p=0.016).

The binding site study demonstrated (see also Figure 2) that CRFR1 was strongly stained (Grade 3) in all but one tissue of Group A, all tissues of Group B, and was found moderately to strongly positive in Group C (Grade 2-3). CRFR2 was also present in all but one tissue of Group A, all tissues of Group B and all but two tissues (male fetus, 23 w, with congenital disorders of visceral cranium/face and female fetus, 27 w, with recessive fetal development) of Group C at varying intensities (Grade 1-3, mostly 2). CRF-BP immunoreactivity was intense (Grade 3) in all fetuses of all Groups and trimesters. No correlation was found between CRF system presence and the presence of chorioamnionitis, maternal age and fetal sex, although the limitations due to the small number of tissues in some groups and sex equilibrium (Group A) hampered statistical analysis (see Table 1).
Figure 1. Immunohistochemistry for CRF neuropeptides and binding sites in heart tissues of human fetuses of different gestational trimesters, with or without diagnosed pathology. Grading (Gr) for the myocytes is shown. Some clearly positive vessels are also shown by arrows. A-G: fetuses with no diagnosed pathology from the second (B, C, E, F, G) and the third trimester (A, D). a-g: pathological fetuses from the first (c), second (a, b, e, f) and the third gestational trimester (d, g), diagnosed with various pathologies other than the heart. Gestational age was estimated by the mother’s LMP. Original magnification: X200. Scale bar = 100 μm. H: negative control (third trimester fetus).

**Grade 3:** >70% positive cells in the total number of cells of the specific cell-type counted per field.

**Grade 2:** 40-70% positively stained cells.

**Grade 1:** 10-40% positively stained cells.

**Grade 0:** <10% positively stained cells.

**Immunohistochemical localization of the CRF system in a fetus with heart pathology**

Among the Group C fetuses, there was one fetus diagnosed with heart pathology that is presented as a case study. Specifically, this fetus was female, 23 weeks old (second gestational trimester) and was suffering from the presence of an interventricular foramen, right ventricle hypertrophy and aortic translocation astride the interventricular septum (Figure 3). CRF, Ucn I, CRFR1 and CRF-BP presence was strongly positive (Grade 3), while Ucn II was moderate (Grade 2) and CRFR2 weak (Grade 1). Ucn III was not detected in the heart tissue of this fetus. It is also noteworthy that in this specific fetus, receptor localization was both cytoplasmic and membranic.

**DISCUSSION**

Given the considerable interest in the role of the CRF system in cardiovascular phenomena and the total lack of available information on its presence in...
Table 2. Semi-quantitative estimation for the presence of CRF, Ucn I, II and III, CRFR1, CRFR2 and CRF-BP detected by immunohistochemistry in the myocytes of human fetal heart tissues. Numbers represent the number of fetuses in each of the four grades (G), in every gestational trimester as estimated by the mother’s last menstrual period (LMP), in the Group A (fetuses with no pathology) and respective p value are shown. n=number of fetuses.

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Grade</th>
<th>Gestational trimester</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>First (Total n: 0)</td>
</tr>
<tr>
<td>CRF</td>
<td>G:0</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>G:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:3</td>
<td></td>
</tr>
<tr>
<td>Ucn I</td>
<td>G:0</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>G:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:3</td>
<td>13</td>
</tr>
<tr>
<td>Ucn II</td>
<td>G:0</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>G:1</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>G:2</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>G:3</td>
<td>2</td>
</tr>
<tr>
<td>Ucn III</td>
<td>G:0</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>G:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:3</td>
<td></td>
</tr>
<tr>
<td>CRFR1</td>
<td>G:0</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>G:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:3</td>
<td>12</td>
</tr>
<tr>
<td>CRFR2</td>
<td>G:0</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>G:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:3</td>
<td></td>
</tr>
<tr>
<td>CRF BP</td>
<td>G:0</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>G:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:3</td>
<td></td>
</tr>
</tbody>
</table>

n: number of fetuses.

the fetal heart, this study aimed to describe the histological mapping of CRF neuropeptides and binding sites in the human fetal heart. Forty archival human fetuses, including fetuses without diagnosed pathology, with chromosomal abnormalities or with congenital disorders were used and antigen localization was studied by immunohistochemistry. Our samples were representative of different gestational ages allowing developmental observations, although the small number of tissues in some groups (first trimester group) limited conclusions. Nevertheless, given the rarity
Table 3. Semi-quantitative estimation for the presence of CRF, Ucn I, II and III, CRFR1, CRFR2 and CRF-BP detected by immunohistochemistry in the myocytes of human fetal heart tissues. Numbers represent the number of fetuses in each of the four grades (G), in every gestational trimester as estimated by the mother’s last menstrual period (LMP), in the Group B (fetuses with genetic disorders).

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Grade</th>
<th>Gestational trimester</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>First (Total n: 0)</td>
</tr>
<tr>
<td>CRF</td>
<td>G:0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:3</td>
<td></td>
</tr>
<tr>
<td>Ucn I</td>
<td>G:0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:3</td>
<td></td>
</tr>
<tr>
<td>Ucn II</td>
<td>G:0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:3</td>
<td></td>
</tr>
<tr>
<td>Ucn III</td>
<td>G:0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:3</td>
<td></td>
</tr>
<tr>
<td>CRFR1</td>
<td>G:0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:3</td>
<td></td>
</tr>
<tr>
<td>CRFR2</td>
<td>G:0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:3</td>
<td></td>
</tr>
<tr>
<td>CRF BP</td>
<td>G:0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:3</td>
<td></td>
</tr>
</tbody>
</table>

n: number of fetuses.

Table 4. Semi-quantitative estimation for the presence of CRF, Ucn I, II and III, CRFR1, CRFR2 and CRF-BP detected by immunohistochemistry in the myocytes of human fetal heart tissues. Numbers represent the number of fetuses in each of the four grades (G), in every gestational trimester as estimated by the mother’s last menstrual period (LMP), in the Group C (fetuses with congenital disorders).

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Grade</th>
<th>Gestational trimester</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>First (Total n: 1)</td>
</tr>
<tr>
<td>CRF</td>
<td>G:0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:3</td>
<td></td>
</tr>
<tr>
<td>Ucn I</td>
<td>G:0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:3</td>
<td></td>
</tr>
<tr>
<td>Ucn II</td>
<td>G:0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:3</td>
<td></td>
</tr>
<tr>
<td>Ucn III</td>
<td>G:0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:3</td>
<td></td>
</tr>
<tr>
<td>CRFR1</td>
<td>G:0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:3</td>
<td></td>
</tr>
<tr>
<td>CRFR2</td>
<td>G:0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:3</td>
<td></td>
</tr>
<tr>
<td>CRF BP</td>
<td>G:0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G:3</td>
<td></td>
</tr>
</tbody>
</table>

n: number of fetuses.

of such sample availability, we considered these data worth presenting. Immunoreactivity for all antigens was found in cardiac myocytes of all groups, in almost all samples, at different levels, except Ucn III that was present in almost half of the fetuses with pathology (both chromosomal and congenital) but was not detected at all in normal fetuses. Ucn III was more often present during the first stages of development and in fetuses with congenital disorders. Among fetuses with pathology there was one fetus diagnosed with heart pathology and it is presented as a case study, revealing the presence of all but Ucn III CRF system antigens. Logistic regression models for the exploration of possible predictors of the presence...
of each factor were not applicable due to the size of our sample, which did not comply with the criteria set by Green SB. The results presented show for the first time the presence of the full CRF system, both neuropeptides and binding sites, in the human fetal heart. This is not surprising, as the presence of CRF neuropeptides and receptors has previously been shown in the human adult heart. Moreover, we have recently demonstrated the full CRF system localization in the lungs of normal and pathological human fetuses and suggested a possible role in its development.

CRF and CRF-BP immunoreactivity was strongly positive (Grade 3), while Ucn I presence was moderately to strongly positive (Grade 2-3) in all fetuses of all Groups and trimesters. It is well established that CRF and Ucn I bind to CRF-BP with higher affinity than to the other binding sites of the CRF system, thus their co-expression points to CRF-BP as a local regulator of their bioavailability.

A previous study using in situ hybridization in order to evaluate CRF mRNA in the rat thorax evidenced expression during embryonic days 12 to 16, peaking on the 14th day, and located especially in the serous membranes, such as the pericardium. The researchers concluded that CRF must play a crucial role in the development of the pericardium. This is also corroborated by our results, although interspe-
cies comparisons to our study cannot be made, as rat embryonic days 12-16 correspond to 28-52 weeks in humans and our sample comprises fetuses of 12-39 weeks of gestation.

Among the fetuses with congenital disorders of our study there was a fetus that was diagnosed with heart pathology. Specifically, this fetus was female, 23 weeks (2nd gestational trimester) and suffered from the presence of an interventricular foramen, right ventricle hypertrophy and aortic translocation astride the interventricular septum (Figure 2). In this fetus, CRF, Ucn I and CRFR1 presence was very strong, while Ucn II and III and CRFR2 were absent or weak. These findings could corroborate with other studies in mice, which have revealed the strong cardioprotective role of Ucn III, via CRFR2, and its smaller, parallel action on the hypothalamic stress axis, since it does not activate CRFR1. Ucn I plays also a cardioprotective role against ischemia reperfusion injury in mice when accompanied by CRFR2b; however, at the same time it activates CRFR1 in the pituitary gland, which causes activation of the hypothalamic stress axis and therefore complicates its possible use in the treatment of cardiovascular diseases. Furthermore, according to other researchers, Ucn I actions regulated by CRFR2 are mainly anti-inflammatory, most probably protecting cardiac myocytes from hypoxic death.

The strong Ucn I presence in the fetus of our study that suffered from right heart hypertrophy can also be correlated with the results of another study group: this showed that Ucn I was present in the cardiac myocytes of the left ventricle of normal adult human hearts and that this immunoreactivity was more intense in the left ventricle of a failing heart than of a normal heart. In addition, in rats with left heart hypertrophy due to DOCA-salt therapy, it was evident that Ucn I mRNA expression was higher, while CRFR2β m-RNA expression was significantly reduced compared to a normal left heart ventricle. The same research group concluded that although the mechanism of increased Ucn I mRNA expression or of increased Ucn I immunoreactivity in left heart hypertrophy or in the failing heart is still unknown, the importance of this fact seems to be correlated, at least partly, with the positive inotropic and hypertrophic action of Ucn I. Either way, the latter has been the main study subject of many other research groups. Moreover, Ikeda et al concluded that Ucn I is secreted not only by the cardiac myocytes but also by non-myocytes and that the primary source of Ucn I that acts on the heart is the heart itself. It was additionally proposed that Ucn I could lead to the proliferation of cardiac myocytes and non-myocytes, thereby causing hypertrophy and fibrosis.

Our results indicate that Ucn III may be more frequently present in embryos of younger gestational age, implying a role of Ucn III in development. In addition, its more frequent presence in fetuses with disorders of organs other than the heart could possibly indicate its involvement in impaired fetal growth. On the other hand, it could be the result of a recessive development, as it was not detected in the only fetus of our study that suffered from congenital disorders of the heart. In general, the changes in Ucn III in Groups B and C could represent a local protective mechanism to stressful stimuli, although we could not draw conclusions on whether the expression changes in the ligands and receptors is a compensatory rather than a causative mechanism. Further studies in larger groups could clarify its involvement in fetal development and pathology, especially as urocortins have recently been identified as a new group of inotropic factors with multiple and important effects on the cardiovascular, hemodynamic, neurohormonal and renal system and possibly novel players in the pathophysiology of the heart and its treatment.

In conclusion, our results present the histological mapping of the full CRF system, both neuropeptides and binding sites, in the heart of normal and pathological human fetuses, at different developmental stages. Although of weak clinical impact, we considered our findings of some importance given the rarity of this type of tissue availability. A more detailed study of the time-course of alterations of the CRF system in the fetal heart could promote a better understanding of its role given that the intracellular and/or organic conditions change dynamically through gestation. Its role in fetal development and fetal pathology awaits further investigation.

ACKNOWLEDGMENTS

We would like to thank Dr. D. Grigoriadis, Neurocrine Bioscience Inc., San Diego, Calif., U.S.A., for kindly providing the antisera for CRFR1.
This research has been co-financed by the European Union (European Social Fund – ESF) and Greek national funds through the Operational Program “Education and Lifelong Learning” of the National Strategic Reference Framework (NSRF) - Research Funding Program: Heracleitus II. Investing in the knowledge society through the ESF.

REFERENCES


Presence of the RET Cys634Tyr mutation and Gly691Ser functional polymorphism in Iranian families with multiple endocrine neoplasia type 2A

Maryam Nasiri Aghdam,1 Mohammad Reza Abbaszadegan,2 Alireza Tafazoli,1 Mohammad Aslzare,3 Zohreh Mosavi3

1Medical Genetics Research Center, Department of Medical Genetics, School of Medicine, Mashhad University of Medical Sciences, 2Division of Human Genetics, Immunology Research Center, Avicena Research Institute, Mashhad University of Medical Sciences, 3Endocrine Research Center, Imam Reza/Ghaem Hospital, School of Medicine, Mashhad University of Medical Sciences; Mashhad, Iran

ABSTRACT
PURPOSE: Multiple Endocrine Neoplasia type 2A (MEN2A) is a complex autosomal dominant inherited syndrome characterized by medullary thyroid carcinoma (MTC), pheochromocytoma and primary parathyroid hyperplasia. In patients with only one or two clinical features, identification of a germ line RET (REarranged in Transfection) mutation is required to make the diagnosis and initiate genetic counseling. METHODS: We analyzed blood DNA from three Iranian families with three generations of MEN2A including 20 affected individuals with MTC and four with pheochromocytoma. RET hotspots were amplified in probands and sequenced for mutation detection. RESULT: The causative mutation in all families was found to be the Cys634Tyr missense substitution. The presence of a functional SNP resulting in Gly691Ser was also detected in exon 11 of 15 affected cases. Four patients showed both of these RET variations. CONCLUSION: Our study shows that the Cys634Tyr missense substitution and the Gly691Ser polymorphism are recurrent in Iranian patients, since our families are unrelated. All asymptomatic carriers of the Cys634Tyr high-risk activating mutation were referred for prophylactic thyroidectomy.

Key words: Cys634Tyr mutation, Gly691Ser polymorphism, Multiple Endocrine Neoplasia type 2A, RET proto-oncogene

INTRODUCTION
The human RET proto-oncogene maps to 10q11.2 and encodes a tyrosine kinase transmembrane receptor.1 The RET protein has three domains including an N-terminal extracellular domain that is a ligand for the GDNF neurotropic molecule, a hydrophobic
transmembrane domain, and an intercellular tyrosine kinase domain. The binding of the GDNF family ligand to RET triggers homo-dimerization of RET and a transformational change in the RET intra-cytoplasmic domain. Germline mutations of the RET proto-oncogene cause the dominant inheritance of multiple endocrine neoplasia type 2A (MEN2A), MEN2B, and familial medullary thyroid carcinoma syndromes. Classical MEN2A is the most common MEN2A variant and in 95% of patients RET germline mutations occur in codons 609, 611, 618, or 620 of exon 10, or in codon 634 of exon 11. Virtually all patients with classical MEN2A present with medullary thyroid carcinoma (MTC) while fewer develop pheochromocytoma (PHEO) and primary parathyroid hyperplasia, the frequency of each depending on the specific RET mutation. According to the categorization of the American Thyroid Association, the ATA-HST “highest risk” includes patients with MEN2B and the RET codon M918T mutation, the ATA-H “high” category includes patients with RET codon C634 mutations, and the ATA-MOD “moderate risk” category includes patients with RET codon mutations other than M918T and C634.

Among all thyroid tumors, MTC has a frequency of 5-10% and only 25% of these cases are categorized as familial type. Since thyroid tumors represent only 1% of all human cancer types, the incidence of MEN2 syndromes is estimated to be very low with approximately 500 to 1,000 MEN2 families existing worldwide. Owing to the rarity of the MEN2 syndromes, the prevalence of different RET mutations in distinct geographic areas is not well defined. As a rule, the clinical presentation of MEN2 syndromes will be more variable when the transforming activity of RET mutation is low. Genetic analysis of the RET proto-oncogene can be performed in those families affected by MEN2A and MEN2B, in cases of sporadic MTC or HSCR, and allows exact molecular diagnosis of the disease.

To the best of our knowledge, this is the first report of genetic screening of families of the Eastern region of Iran for identification of MEN2A families. We checked all mutation hotspots of the RET proto-oncogene in exons 11, 10, 13, and 8 by direct sequencing to produce reliable results.

METHODS

Family Selection and Ethics Statement

Three unrelated families who were affected by MEN2A (all from the Khorasan province, Iran) were included in this study after biochemical assays and clinical diagnosis by two endocrine specialists from the Endocrinology Research Center of Mashhad University of Medical Sciences (MUMS). The inclusion criteria for MEN2A phenotype was in line with the guidelines of the American Thyroid Association guidelines on MTC management. All the patients signed an informed consent to genetic analysis approved by the MUMS ethics committee according to the Declaration of Helsinki (1964). In cases of non-availability of patients, the required information was requested from at least two adult family members and hospital records were checked when available. Parents were asked to sign consents for children under the age of 15 years. Clinical features were evaluated over an average of 5 years of follow-up.

Amplification of RET hotspots

Genomic DNA was prepared from peripheral blood leukocytes by standard procedures. DNA samples were amplified from RET exons 8, 10, 11, and 13 by polymerase chain reaction (PCR) using a thermal cycler and specific primers. Nucleotide sequences of the primers were as follows: exon 8, 5′ TTGGGCACTAGCTG-GACG 3′ and 5′ ACCTTCCCAAGTCCAGAGT 3′; exon 10, 5′ AGGCTGAGTGGGCTACGTCTG 3′ and 5′ GTTGAGACCTCTGTGGGGCT 3′; exon 11, 5′ ATGAGGCAGAGCATACGCAGCC 3′ and 5′ CTTGAAGGCATCCACGGAGACC 3′; exon 13, 5′ AACTTGGGCAAGGCGATGCA 3′ and 5′ CTTGAAGGCATCCACGGAGACC 3′.

The PCR for the sequencing was performed in a volume of 50 µl containing 0.5 µM of each oligonucleotide primer, 50 ng of DNA, 1×PCR buffer, 250 µM dNTP, and 2.5 U of Taq polymerase using an automated thermal cycler (Techne, Flexigene, UK). The PCR was started with 5 minutes of pre-denaturation at 95°C, followed by 35 cycles of 40 seconds at 95°C, 30 seconds at 62°C, 55°C, and 64°C for exons 8, 10, 11, and 13, respectively, then 40 seconds at 72°C; lastly, the procedure was completed with 10 minutes at 72°C for the final extension. Following PCR, the amplicon sizes were analyzed in 2% agarose gel and the products
were visualized by green viewer staining. The PCR products were subjected to direct cycle sequencing and restriction enzyme analysis. The sequencing results were aligned to the RET reference sequence with Sequencher version 5.1 software.

**Restriction fragment length polymorphism**

The RFLP analyses of the RET cys634Tyr mutation was performed using Rsal restriction enzymes (Jena Bioscience, Germany) for genotyping of family members. Briefly, the restriction digestion was carried out at 37°C for 2 hours. Each reaction mixture contained 8 µl of the 333 base pair PCR product, 0.1 µl of Rsal (1 U), 2 µl of enzyme buffer, and 9.9 µl of distilled water to make a final volume of 20 µl for mutation identification. The digestion products were analyzed in 3% agarose gel stained with green viewer for RET gene fragments, respectively.

**RESULTS**

Among 20 patients belonging to three families with three generations of MEN2A, four (20 %) presented with PHEO. There were 11 females and 9 males with a mean age of 31 years. The diagnosis of PHEO was made after medullary thyroid carcinoma (n=3, 75%) and before MTC (n=1, 25 %). The family pedigrees are shown in Figures 1, 2, and 3.

Blood samples of 42 family members were collected and DNA extracted for genetic analysis. RET hotspots including exon 8, 10, 11, and 13 were amplified in 3 index cases (A-III1, B-III6 and C-III2) and

![Figure 1. Pedigree of family A. Proband is shown by left arrow. All members with Roman numerals are genetically analyzed. Cases IV6 and V2 were recommended for prophylactic thyroidectomy. Sequencing results illustrated Cys634Tyr mutation and Gly691Ser polymorphism in exon 11 of proband.](image-url)
products were analyzed in 2% agarose gel stained with green viewer. DNA sequence analysis for 4 exons of the RET gene revealed a transversion of TGC-to-TAC at codon 634 of exon 11 resulting in cysteine to tyrosine amino acid change. Additionally, we detected a Gly691Ser polymorphism in exon 11 of all the probands.

We then studied exon 11 in the family members using both RFLP and direct sequencing. RFLP was designed for Cys634Tyr mutation detection using RsaI endonuclease. RsaI cut the wild sequence to 308 and 25 base pairs where it cut the mutated sequence into two locations and made three bands of 230, 77, and 25 base pairs. Results from 3% agarose gel are shown in Figure 4.

The sequencing of exon 11 in the probands and the family members who showed a mutation confirmed the result obtained from the RFLP test. According to the sequencing result, in family A, cases A-IV₆ and A-V₂ were asymptotic Cys634Tyr mutation carriers who were unaware of their condition. Genetic counseling was given to these cases. Case IV-A₆ showed only a palpable thyroid. We found Gly691Ser polymorphisms in cases A-III₁, A-III₇, A-III₉, A-IV₁, A-IV₁₁, A-IV₁₃, A-IV₁₈, and A-V₁. Mild goiter and hypertension is clinical features of cases A-IV₁, A-IV₁₁, and A-IV₁₈, while cases A-IV₁₃ and A-V₁ showed only a palpable thyroid. The proband of family A is a 53 year-old woman who has both a Cys634Tyr mutation and the Gly691Ser polymorphism in exon 11. She presented MTC at the age of 24 and PHEO at the age of 35. In family B,
case number B-IV4 is a 6 month-old asymptomatic son carrying the Cys634Tyr mutation. The other two children of the B-III6 index case including B-IV1 and B-IV2 are carriers of the Gly691Ser polymorphism. The proband is a 33 year-old woman who presented with PHEO one year before MTC at the age of 29. In family C, three carriers of the Cys634Tyr mutation including two asymptomatic cases (C-III7 and C-IV1) were found. Case number C-III7 exhibited hypertension and mild goiter. The Gly691Ser polymorphism was detected in cases C-III1, C-III2, C-III7, and C-III9. Both C-III2 and C-III7 cases showed co-presentation of Cys634Tyr and Gly691Ser substitutions. One known silent mutation (rs1800861) was also detected in exon 13 of the members of families A and C.

Figure 3. Pedigree of family C. Proband is shown by left arrow. All members with Roman numerals are genetically analyzed. Cases III7 and IV4 were recommended for prophylactic thyroidectomy. Sequencing results illustrated Cys634Tyr mutation and Gly691Ser polymorphism in exon 11 of proband.

Figure 4. RFLP results of family C using RsaI endonuclease.
Among a total of 42 genetically analyzed cases, 13 showed the Cys634Tyr high-risk activating mutation, 15 showed the Gly691Ser polymorphism, and four cases showed both of them. Five asymptomatic Cys634Tyr mutation carriers including A-IV6, A-V2, B-IV4, C-III7, and C-IV1 were referred for prophylactic thyroidectomy. All the sequencing results in the three index cases and five mutation carriers were repeated by reverse primer reading.

DISCUSSION

Mutations in the RET proto-oncogene have been implicated in the malignant transformation of parafollicular cells (C cells) of the thyroid that originate from the neural crest during embryogenesis. Germline mutations in RET cause Hirschsprung disease, which is a congenital defect of the enteric nervous system in the hindgut, and MEN2. Mutations converting the RET proto-oncogene into a dominant transforming gene is responsible for tumor components of MEN2A. Allelic imbalance through a tandem duplication and the resulting amplification of mutated RET has been proposed as a possible mechanism of tumor initiation in some patients with MEN2A-related MTC and pheochromocytoma. In most cases of MEN2A, thyroid carcinoma is the first clinical feature, which makes it difficult to diagnose between familial MTC cases and MEN2A without genetic testing. An Iranian MEN2A family first studied by Dr. Moosavi et al in 1992 displayed only MTC in 15 affected family members for many years. In cases with the Cys634Arg mutation show more frequent and early metastases when compared to cases with Cys634Tyr. Mutations in codon 634 have a higher potency of cell neoplastic transformation than those in codons 609 and 611. This finding may be due to modulations in expression of mature RET-encoded protein receptors in the cell membrane. Thus, most patients with MEN2 who harbor RET 634 mutations will have adrenal and parathyroid tumors in addition to thyroid tumors, whereas patients with 609 or 611 mutations will present only with thyroid cancer. Codon-specific RET mutations may also have roles in tissue-specific sensitivity. Therefore, there is a high sensitivity in thyroid tissue, intermediate in the adrenals, and low in parathyroid glands. RET polymorphisms and haplotypes are believed to be genetic modifiers and might be associated with an increased relative risk for the development of disorders. The role of the RET variant allele G691S in MTC has been controversial. The G691S missense polymorphism might alter the function of the protein through creation of a new phosphorylation site affecting downstream signaling or changing the secondary structure of RET. It may be possible that the G691S variant has a role in disruption of topological chromatin domains if it presents a gene-enhancer activity. A recent meta-analysis concluded that the G691S increases the risk of several cancer types, including MTC, via a recessive mechanism of action. Some evidence shows that the RET variant G691S is a disease modifier in sporadic MTC.

It has been suggested that the age of onset of MEN2A can be modified by RET G691S and S904S polymorphisms. Results from a study of Borrello M. (2011) demonstrated that, although RET-G691S is not oncogenic, it enhances the transforming activity of the RET-K666E mutant, hence suggesting a modifier role for this functional polymorphism. Because the transition from C-cell hyperplasia to node-negative and ultimately node-positive MTC takes time, the aforementioned histopathological phases are separated by time intervals. The time lag between malignant transformation and tumor cell spread represents a “window of opportunity” for surgical intervention before the tumor extends beyond the confines of the thyroid gland rendering it harder to cure. For carriers of RET mutations in codon 634, this time interval has been estimated to be around 6.6 years based on the mean age difference between patients with node-negative (10.2 years) and patients with node-positive thyroid cancer (17.1 years). Effective clinical interventions after appropriate genetic counseling are available for prevention through prophylactic thyroidectomy, and, if needed, adrenalectomy and parathyroidectomy. In conclusion, our study showed the presence of the Cys634Tyr mutation and Gly691Ser polymorphism in three Iranian families. Five asymptomatic cases with the Cys634Tyr were unaware of their condition and were referred for prophylactic thyroidectomy.
ACKNOWLEDGEMENTS

This research was funded by Mashhad University of Medical Sciences. We are grateful for the generous assistance of Farzaneh Mohebi, nursing head of Imam Reza Hospital. We also thank all patients and family members for their patience and confidence.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

ETHICAL APPROVAL

All procedures performed in this study were in accordance with the ethical standards of the ethical committee of Mashhad University of Medical Sciences and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

REFERENCES


The growth endocrine axis and inflammatory responses after laparoscopic cholecystectomy

Themistoklis Floros,1 Anastassios Philippou,2 Dimitrios Bardakostas,1 Dimitrios Mantas,1 Michael Koutsilieris2

12nd Propaedeutic Department of Surgery, Laiko General Hospital, 2Department of Experimental Physiology, Medical School, National and Kapodistrian University of Athens; Athens, Greece

ABSTRACT

OBJECTIVE: It is well known that conventional surgery leads to detrimental immune and catabolic responses, thus there is growing interest in the effect of minimally invasive techniques on postoperative endocrine and immune function. The aim of this prospective study was to evaluate the growth hormone (GH)/insulin-like growth factor-1 (IGF-1)/IGF binding protein-3 (IGFBP-3) axis and acute phase (interleukin-6, IL-6, and C-reactive protein, CRP) responses in patients who underwent laparoscopic cholecystectomy. DESIGN: Twenty-nine patients (16 women, 13 men; age: 58±8 years) with a history of uncomplicated symptomatic cholelithiasis participated in the study. Blood samples were collected prior to and at 24 hrs and 48 hrs after laparoscopic cholecystectomy. Serum concentrations of GH, IGF-1, IGFBP-3, and IL-6 were determined by standard sandwich enzyme-linked immunosorbent assay (ELISA), while CRP was measured by nephelometry. ANOVA with repeated measures and Tukey’s post-hoc test were used to evaluate changes in serum measurements. RESULTS: The laparoscopic cholecystectomy resulted in a significant postoperative increase in circulating levels of IL-6 (p=0.031), which is the main cytokine responsible for inducing the acute inflammatory response, and of the acute phase protein CRP (p=0.005). A significant increase in GH levels at 24 hrs (p=0.034) and decrease of IGF-1 on both postoperative days were also found (p=0.045, 0.044), while no changes were documented in IGFBP-3 levels. Significant correlations were revealed between postoperative levels of the acute phase proteins and growth axis hormones (p<0.05 - 0.001). CONCLUSIONS: Our findings suggest that laparoscopic cholecystectomy induces acute phase endocrine and immune responses. These changes may represent a state of systemic inflammation and GH resistance, compatible with possible cytokine-induced anti-anabolic or catabolic effects even after this minimally invasive cholecystectomy.

Key words: Acute phase response, Growth hormone, Inflammation, Insulin-like growth factor-1, Interleukin-6, Laparoscopy

Address for correspondence:
Themistoklis Floros, MD, 2nd Propaedeutic Department of Surgery, Laiko General Hospital, Medical School, National and Kapodistrian University of Athens, Ag. Thoma 17, Goudi-Athens, 11527 Greece; Tel.: +30 2106138964, Fax: +30 2132061764, E-mail: jfloros@otenet.gr

Received: 19-06-2015, Accepted: 22-10-2015
INTRODUCTION

Over the last decade, an increasing number of studies have been conducted to investigate the effects of minimally invasive surgical techniques on surgical stress response and a variety of immune function parameters.1-4 The introduction of advanced laparoscopic methods has revealed several clinical advantages of minimally invasive surgery,1,3,5 including less postoperative pain, quicker recovery, and shorter hospital stay.2,6-8

Nevertheless, although laparoscopic techniques are being increasingly used in the management of various intra-abdominal conditions, there is conflicting evidence regarding the effect of laparoscopic surgery on postoperative systemic inflammatory response. Specifically, concern has been raised that increased intra-abdominal pressure during laparoscopy may promote bacteremia and systemic inflammation,5,9 while recent findings suggest that carbon dioxide pneumoperitoneum could have a protective effect against postoperative enhanced systemic inflammation, particularly after laparoscopic cholecystectomy.3,10,11

Immunity and inflammation after surgery are mediated by cytokines, such as tumour necrosis factor-α (TNF-α), interleukin (IL)-1, and IL-6, which are activated as an early response to tissue injury.12-14 Specifically, IL-6 is the main cytokine responsible for inducing the systemic changes known as the acute phase response, which includes the production of acute phase proteins in the liver, such as C-reactive protein (CRP), and plays a major role in inflammation.14-17

Moreover, surgical stress response is characterized by increased secretion of pituitary hormones, decreased secretion or effects of anabolic hormones, and hypermetabolism. The overall metabolic effect of the tissue injury-induced endocrine and metabolic changes is increased catabolism, and those changes are thought to mediate the increased demand on the reserves and immune competence of patients post surgery.2,14,18 In particular, growth hormone (GH) is secreted by the anterior pituitary and its secretion increases in relation to the severity of tissue injury after surgery.14 Many GH actions are mediated by insulin-like growth factor-1 (IGF-1), which, in the context of its endocrine activity, is produced and secreted by the liver, skeletal muscle, and other tissues in response to stimulation by GH.19-21 Furthermore, it has been proposed that acute inflammation induces a GH-resistant state as part of a regulatory mechanism of the body to restrict growth and energy storage, in which the elevated GH secretion is not followed by increased circulating IGF-1 levels.22-24

Although the effect of surgical stress and/or inflammatory responses on endocrine function after laparoscopic surgery has been described in humans,2,13,25-27 studies designed to detect the relation between the response of the anabolic GH/IGF-1 axis and laparoscopic cholecystectomy in the context of an acute phase response are lacking. This prospective study aimed at determining whether there are acute phase changes in growth axis hormones in patients who underwent laparoscopic cholecystectomy.

MATERIALS AND METHODS

Ethical approval

All experimental procedures were in accordance with the ethical standards of the Ethics Committee of the Institutional Review Board of “Laiko” General Hospital, which approved the study, and with the Declaration of Helsinki. Written informed consent was provided by all volunteers participating in this study.

Patients

From September 2013 to May 2014, a hospital-based prospective study was conducted in 29 consecutive patients (16 women, 13 men; age 58±8 years). They had a history of uncomplicated symptomatic cholelithiasis and were referred to the authors’ department for surgical treatment of their disease. The patients were scheduled for laparoscopic cholecystectomy and had not received any treatment for at least a week before they underwent the surgical excision of the gallbladder. In all patients, general anesthesia was induced with propofol or midazolam, while postoperative analgesia consisted of routine administration of fentanyl or paracetamol. The choice of anesthetics was determined by the attending anesthesiologists. Patients were released from hospital on the third postoperative day.

Blood sampling and serum measurements

Blood samples were collected between 8:00 and 9:00 am from each individual patient before the op-
Growth hormone axis responses after cholecystectomy

A one-way analysis of variance (ANOVA) with repeated measures over time was employed to evaluate changes in all serum measurements (SPSS v. 21 statistical package; SPSS Inc., Chicago, IL, USA). Where significant F ratios were found for main effects (p < 0.05), the postoperative mean values were compared with the preoperative means (control) using Dunnett’s post hoc tests. Relationships between variables were examined using Pearson’s correlation coefficient (r). All data are presented as mean ± SE. Statistically significant changes were considered at p<0.05.

RESULTS

The coefficient $r^2$ for standard curves of all the ELISA analyses was 0.994-1. Analysis of the inflammation-related factor IL-6 in the blood revealed that the laparoscopic cholecystectomy resulted in a significant three-fold increase of circulating IL-6 levels on postoperative day 2 (48 hrs) compared to the preoperative (PRE) levels (17.9±4.6 vs. 5.2±1.9 pg/ml; F ratio: 3.142, p=0.045, Table 1). Similarly, there was a significant five-fold increase in the serum levels of the acute phase protein CRP on postoperative day 2 compared to the preoperative levels (20.9±5.6 vs. 3.87±0.28 mg/L; F ratio: 4.965, p=0.010, Table 1). A significant two-fold increase was observed in the levels of GH on postoperative day 1 (24 hrs) before returning to the preoperative levels on day 2 (PRE levels: 520.6±141.2 vs. 1100.9±216.1 vs.

Table 1. Serum concentrations of the growth axis hormones GH, IGF-1, and IGFBP-3, and of the acute phase proteins IL-6 and CRP, preoperatively (PRE) and on days 1 and 2 post laparoscopic cholecystectomy. P values refer to comparisons with the preoperative concentration (mean ± SE)

<table>
<thead>
<tr>
<th></th>
<th>PRE</th>
<th>Day 1</th>
<th>Day 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH (pg/ml)</td>
<td>520.6±141.2</td>
<td>1100.9±216.1</td>
<td>505.6±142.8</td>
</tr>
<tr>
<td>p=0.034</td>
<td>(p=0.997)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-1 (ng/ml)</td>
<td>130.9±12.3</td>
<td>110.6±13.2</td>
<td>110.5±11.1</td>
</tr>
<tr>
<td>p=0.045</td>
<td>(p=0.044)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGFBP-3 (pg/ml)</td>
<td>1.95±0.14</td>
<td>1.96±0.13</td>
<td>1.96±0.14</td>
</tr>
<tr>
<td>p=1.000</td>
<td>(p=0.997)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>5.2±1.9</td>
<td>11.9±3.0</td>
<td>17.9±4.6</td>
</tr>
<tr>
<td>p=0.319</td>
<td>(p=0.031)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>3.87±0.28</td>
<td>10.76±2.56</td>
<td>20.9±5.6</td>
</tr>
<tr>
<td>p=0.368</td>
<td>(p=0.005)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Circulating IGF-1 levels were significantly decreased on both postoperative days (PRE levels: 130.9±12.3 vs. 110.6±13.2 vs. 110.5±11.1 ng/ml; F ratio: 3.766, p=0.031, Table 1), while no changes were observed in serum levels of IGFBP-3 throughout the experimental period (PRE levels: 1.95±0.14 vs. 1.96±0.13 vs. 1.96±0.14 μg/ml; F ratio: 0.002, p=0.998, Table 1). Correlation analyses showed significant associations between the circulating levels of the acute phase proteins and the growth axis hormones, as shown in Table 2 (p <0.05-0.001).

**DISCUSSION**

Several aspects of postoperative endocrine responses have been described after minimally invasive surgery; however, there is little information regarding the effect of laparoscopic cholecystectomy on the growth endocrine axis. In this prospective trial, we evaluated changes in circulating GH/IGF-1 axis proteins in patients who underwent laparoscopic cholecystectomy. The main finding of the study is that even after this minimally invasive surgery, acute phase responses of the growth endocrine axis are induced, as indicated by the postoperative changes in the circulating levels of IL-6, CRP, GH, and IGF-1.

Changes in circulating levels of IL-6 precede the increase in serum concentration of other acute phase proteins, such as CRP, that act as inflammatory mediators. The study of these two inflammation biomarkers, and particularly of IL-6, allows direct quantification of the acute phase inflammatory response, as IL-6 and IL-1 are major activators of the cell-mediated immune system response. IL-6 production (as well as that of CRP), which seems to be the most indicative of the severity of tissue injury, is normally moderate in a minimally invasive procedure such as laparoscopic surgery.

In the present study, a gradual increase in the circulating levels of both IL-6 and CRP was observed on postoperative days 1 and 2, which became significant 48 hrs after the laparoscopic removal of the gallbladder, while high positive correlations were also observed between the postoperative levels of these acute phase factors, indicating their similar responses following the laparoscopic surgery. Similarly, transient or sustained increases in IL-6 and CRP levels have previously been reported after laparoscopic cholecystectomy, indicating the induction of an acute phase inflammatory response following this minimally invasive surgery.

The inflammatory cytokine actions are mediated, at least partially, by indirect changes in the activity of growth promoting hormones such as GH and IGF-1. The stress response to surgery is comprised of a number of hormonal changes induced by neural activation of the hypothalamic-pituitary axis. The duration and magnitude of this response is proportional to the tissue

---

**Table 2.** Significant correlations revealed between the circulating levels of the growth axis hormones GH, IGF-1, and IGFBP-3, and the acute phase proteins IL-6 and CRP, preoperatively (PRE) and on days 1 and 2 post laparoscopic cholecystectomy

<table>
<thead>
<tr>
<th></th>
<th>CRP PRE</th>
<th>CRP Day 1</th>
<th>CRP Day 2</th>
<th>IL-6 Day 1</th>
<th>IL-6 Day 2</th>
<th>BP-3 Day 1</th>
<th>BP-3 Day 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH</td>
<td>r=0.749 (p&lt;0.001)</td>
<td>r=0.803 (p&lt;0.001)</td>
<td>r=0.444 (p=0.020)</td>
<td>r=0.571 (p=0.001)</td>
<td>r=0.540 (p=0.002)</td>
<td>r=0.790 (p&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td>IGF-1</td>
<td>r=-0.477 (p=0.025)</td>
<td>r=-0.471 (p=0.027)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP-3</td>
<td>r=0.431 (p=0.040)</td>
<td>r=0.484 (p=0.012)</td>
<td>r=0.395 (p=0.041)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>r=0.887 (p&lt;0.001)</td>
<td>r=0.860 (p&lt;0.001)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
injury and has an overall catabolic effect on stored body fuels. More specifically, IGF-1 is implicated in a vast number of physiological functions related to tissue growth, development, and metabolism. Trauma and conventional surgery result in a state of acquired GH resistance, characterized by high GH levels, decreased anabolic response to GH administration, and reduced levels of its main effector, IGF-1. It is thought that the acute reduction in circulating IGF-1 levels reflects suppression of the GH/IGF-1 axis in response to the acute stress of surgery.

In this study, decreased levels of circulating IGF-1 were found on both postoperative days after laparoscopic cholecystectomy. This decrease was accompanied by a transient increase in GH levels on the first postoperative day, indicating possible growth hormone axis suppression similar to that described after conventional surgery. In addition, significant positive correlations were revealed between the postoperative response of acute phase proteins and GH, as well as inverse correlations between CRP and IGF-1 pre- and postoperative levels. Overall, these relationships may reflect a regulatory effect of IL-6 and CRP on growth axis hormones compatible with a state of systemic inflammation and GH resistance. Moreover, the postoperative correlations found between acute phase proteins and IGFBP-3 may suggest a compensatory systemic response of IGFBP-3 to increase the diminished IGF-1 levels via acute inflammation. The influence of anesthesia could contribute to these stress-induced postoperative changes in GH/IGF-1 levels, although a persistent, lasting more than two days, and not transient increase in GH levels has been reported after major surgical trauma. To our knowledge, this is the first study showing concurrently the acute phase changes in GH/IGF-1 axis hormones after this minimally invasive cholecystectomy and expands on previous evidence that laparoscopic cholecystectomy can induce an acute endocrine stress response.

Alterations in cytokines and endotoxin may decrease GH receptor availability in the liver, leading to reduced levels of circulating IGF-1, while the decreased levels of IGF-1 may result in increased GH secretion due to the lack of negative (inhibitory) feedback of IGF-1. Taking into consideration the acute inflammatory response observed in the present study, it can be speculated that the GH/IGF-1 responses may represent cytokine-induced anti-anabolic or catabolic effects even after laparoscopic cholecystectomy. Furthermore, neuroendocrine stress response and inflammatory response, activated by afferent impulses from the site of tissue injury to the brain, occur following laparoscopic cholecystectomy. Central administration of IGF-1 can counteract the activity of pro-inflammatory cytokines produced in the brain; however, its decreased levels observed in this study are not likely to attenuate a neuro-inflammatory response.

Although it was previously postulated that circulating or locally produced IGF-1 mediates the growth-promoting actions of GH, it has since been established that GH and IGF-1 can also act independently of each other. Furthermore, investigation into IGF-1 and IGFBP-3 has been used for the evaluation of GH/IGF-1 axis disorders. Biological actions of IGF-1 are modulated by a family of at least six IGFBPs, as they bind IGF-1 and increase its half-life in the circulation. IGFBPs, and particularly IGFBP-3, can modulate, both in the circulation and the extracellular environment, the extent of IGF-dependent effects via the regulation of free IGF-1 concentration and its local bioavailability in the tissue.

After major surgery there are complex and diverse changes in IGF-1 and IGFBPs. Thus, a larger proportion of circulating smaller fractions of the IGF-1/IGFBP-3 complex has been reported after major conventional surgery, possibly as a result of proteolytic modification of IGFBP-3 to increase the bioavailability of IGF-1. In the present study, similarly to previous findings reported after laparoscopic colectomy, we did not observe any significant changes in the serum levels of IGFBP-3 on the two consecutive postoperative days after laparoscopic cholecystectomy, suggesting that minimally invasive cholecystectomy does not affect the circulating levels of this growth hormone axis protein. Interestingly however, we demonstrated high correlations between IGFBP-3 and IGF-1 levels on both postoperative days, indicating a possible regulatory effect of IGFBP-3 on circulating IGF-1 postoperatively.
In conclusion, concurrent investigation of the changes in GH/IGF-1 axis hormones after laparoscopic cholecystectomy revealed that this minimally invasive procedure induces acute phases responses in the growth endocrine axis. It remains to be confirmed whether these responses reflect possible cytokine-induced anti-anabolic or catabolic effects even after this minimally invasive surgery.

REFERENCES


Progesterone pretreatment increases the stress response to social isolation in ewes

Aline Freitas-de-Melo,1 Juan Pablo Damián,1 Maria José Hötzel,2 Georgget Banchero,3 Rodolfo Ungerfeld4

1Departamento de Biología Molecular y Celular, Facultad de Veterinaria, Universidad de la República, Lasplaces, Montevideo, Uruguay; 2Laboratório de Etologia Aplicada, Departamento de Zootecnia e Desenvolvimento Rural, Universidade Federal de Santa Catarina, Rodovia Admar Gonzaga, Florianópolis, Brazil; 3Instituto Nacional de Investigación Agropecuaria, La Estanzuela, Colonia, Uruguay; 4Departamento de Fisiología, Facultad de Veterinaria, Universidad de la República, Lasplaces, Montevideo, Uruguay

ABSTRACT

OBJECTIVE: In rodents, progesterone (P4) pretreatment increases anxiety and response to stressors. Social isolation is a stressor that generates physiological and behavioural stress responses in sheep. The aim of the study was to compare the stress response of anoestrous ewes previously treated or not with P4 to the social isolation test. DESIGN: Ten ewes received P4 treatment during 13 d (group P4-W) and another 10 remained untreated as controls (group Con). The ewes were individually isolated in a novel place during 10 min, 24 h after the end of P4 pretreatment and their behaviours were recorded. Cortisol and P4 concentrations as well as body surface temperature were recorded before and after the test. RESULTS: Ewes of the P4-W group presented higher cortisol levels 0, 10, 20 and 30 min after the social isolation and had greater area under the curve of cortisol compared to Con ewes (41,785%±4,156% vs. 25,682%±4,565% during 75 min). Progesterone and body surface temperature increased after social isolation, with no differences between P4-W and Con ewes. There were no differences in behavioural responses to social isolation. CONCLUSIONS: P4 pretreatment appears to augment the stress response to social isolation in anoestrous ewes.

Key words: Behaviour, Body temperature, Cortisol, Open field test, Sheep

INTRODUCTION

Progesterone (P4) reduces the stress response in rodents through neuroactive metabolites that contain the 3α-hydroxy group.1,2 These metabolites produce anxiolytic and sedative effects through binding to γ-amino butyric acidA (GABA_A) receptor,3 thereby reducing the neuroendocrine and behavioural response to stressors.4,5 In ewes, P4 administration reduces the behavioural and physiological responses to weaning, a strong stressor.6 On the other hand, it has also been reported that reduction of P4 in rodents – as is observed immediately after luteolysis – has anxiogenic effects through a decrease in the inhibitory effect of...
Thus, the animals have an increased sensitivity to stressors, as for example the open field test. Crossley et al demonstrated that these metabolites bind to the GABA	extsubscript{A} receptor of central nervous system tissue of sheep in vitro, indicating that this could be the pathway of its anxiolytic and anxiogenic effects.

As sheep are gregarious animals, individual isolation from the flock provokes an important stress response. Therefore, the open field test is a standardized test used to evaluate the stress response of sheep to social isolation. In this test, sheep are individually introduced into an unfamiliar and isolated environment for a certain period of time. Socially isolated sheep show behavioral responses indicative of stress, such as an increase in locomotion, the number of vocalizations, urinations and defecations and a reduction in the number of exploratory behaviors, as well as increases in cortisol concentration and rectal temperature. Although an increase in P4 concentration has not been observed in response to social isolation, it has been reported that P4 in ewes increases in response to ACTH administration and after transportation. Considering all this information, ewes should respond strongly to stressors after P4 pretreatments. Taking all the above into consideration, ewes should be more sensitive to stressors after traditional hormonal treatments applied for estrous synchronization and artificial insemination, given that the P4 levels (and thus its neuractive metabolites) change acutely following this procedure. We hypothesized that anoestrous ewes are more sensitive to social isolation after P4 concentrations have been withdrawn. Thus, the aim of the study was to compare the stress response to social isolation of anoestrous ewes previously treated or not with P4.

**EXPERIMENTAL ANIMALS AND METHODOLOGY**

**Location and animal management**

All the procedures were approved by the Comisión Honoraria de Experimentación Animal of the Facultad de Veterinaria, Universidad de la República, Uruguay. The study was performed at Estación Experimental La Estanzuela of the Instituto Nacional de Investigación Agropecuaria (INIA), Uruguay (34°28’S) during the non-breeding season (September-October, spring). Twenty nulliparous Polwarth ewes that weighed 40.3±0.8 kg (mean±SEM) and had a body condition score of 2 (median, min=1.75; max=2.5; in a 1 to 5 scale, where 1=emaciated and 5=obese) were used in the experiment. Ewes were assigned to two experimental groups (n=10 each), which grazed the same pasture of white clover and lucerne, in separated paddocks (50 m x 25 m), with free access to water. All ewes were shorn 20 d before the experiment started.

**Experimental treatments**

Both experimental groups were homogeneous for body weight and body condition score. While 10 ewes received an intravaginal P4 releasing device [Controlled Internal Drug Releasing (CIDR), 0.3 g P4; Pfizer, Auckland, New Zealand] for 13 d (group P4-W), the other 10 ewes remained as untreated controls (group Con). To avoid the effects of acute change of housing interfering with experimental results and to make it easier to catch and sample the ewes, 3 d before the test was performed all the ewes were moved to two pens (10m×10m) located 30 m from the place where the social isolation test was going to be performed. During those days the ewes received lucerne hay and water ad libitum.

**Progesterone profile group**

To characterize the P4 profile during and after CIDR treatment while avoiding excessive management of the experimental ewes, we collected blood samples from another 5 ewes (body weight = 40.2±1.80 kg; body condition score ranged from 2 to 2.25) from the same flock, which were managed similarly. These ewes received a CIDR for 13 d (P4-profile group) and were bled by jugular venipuncture on Days 0, immediately before CIDR insertion (Day 0=CIDR insertion), 1, 2, 3, 7, 10 and 13 during CIDR treatment, and 4, 8, 20 and 26 h after CIDR withdrawal.

**Social isolation test**

The ewes were individually moved to the social isolation pen where they remained for 10 min. After this test, the ewes were moved to another pen (6.0m×6.0m) where other ewes from the same flock were housed and where they remained during the entire sampling period. The social isolation pen measured
3.0m×3.5m, 1.5m high, and had black walls. The floor was painted to mark 16 squares of equal size. During the social isolation test, the ewes had no visual, olfactive or auditive interactions with humans or the other animals, which remained at a minimum distance of 20 m.

The social isolation test was performed 24 h after CIDRs were withdrawn in the P4-W ewes. CIDRs were placed in 5 animals for each treatment on day one and on another 5 the following day and likewise withdrawn also with 24 h of difference to homogenize the time from CIDR withdrawal and avoid differences in the time of the day in which the ewes were tested. Therefore, the tests were performed during two consecutive days, thus testing 5 ewes from each group each day. All tests were performed between 08:00 h and 13:00 h. The ewes were tested at intervals, one from each group at a time. Two ewes, one from each group, escaped from the pen during the test so their data were not considered.

### Blood samples

Blood samples were collected by jugular venipuncture 11 and 2 d before CIDR insertion to confirm the anoestrous condition with P4 concentrations. Samples were also taken 5 min before the test, immediately after (0 min) and 10, 20, 30, 45 and 60 min after the test. Blood samples were centrifuged at 1500 x g for 10 min and the serum was separated and frozen at -20°C.

### Cortisol and progesterone measurements

Cortisol concentration was determined on serum from all samples collected before and after the social isolation test. Serum cortisol concentration was measured at the Laboratorio de Técnicas Nucleares, Facultad de Veterinaria, Montevideo, Uruguay, using a solid-phase radioimmunoanalysis kit (Coat-a-Count, Siemens, Los Angeles, CA, USA). The analytical sensitivity of the assay was 19.04 nmol/L and the intra-assay coefficient of variation was 11.7%.

Progesterone concentration was measured on serum samples collected 11 and 2 d before CIDR insertion to confirm the anoestrous status of the ewes, considering P4 concentration >3.2 nmol/L as luteal value 19. Progesterone concentration was measured on the samples collected 5 min before and immediately after the social isolation test and 30 min later. Progesterone was also measured on all serum samples collected in the P4-profile group of the ewes. Serum P4 concentration was measured at the Laboratorio de Técnicas Nucleares, Facultad de Veterinaria, Montevideo, Uruguay, using a solid-phase radioimmunoanalysis kit (Coat-a-Count, Siemens, Los Angeles, CA, USA). The analytical sensitivity of the assay was 0.25 nmol/L and the intra-assay coefficient of variation was 10.7%.

### Body surface temperature

Surface temperatures were recorded on the skin of the left thorax and abdominal areas, in the medial region, using a laser thermometer (IR-102 Infrared Thermometer, Super Elec. Equip. Co, China), simultaneously to blood collection, until 45 min after the social isolation test.

### Behavioural recordings

Two digital cameras recorded the ewes’ activities continuously during the social isolation. The following variables were analyzed from the videos: the number of lines crossed, vocalizations, eliminations (urinations + defecations), sniffing, escape attempts, freezing, latencies to first vocalization and elimination and the time that each ewe remained on the periphery squares.

### Statistical analysis

The percentages at which cortisol and P4 concentration changed were determined considering the initial value (before the social isolation test) as 100%. The area under the curve for cortisol and P4 were calculated using GraphPadPrism Demo (GraphPad Software, Inc., San Diego, USA). Cortisol concentrations of P4-W and C ewes before the social isolation test were compared by ANOVA. Body surface temperature and the percentages at which cortisol and P4 concentrations changed were compared between treatments with the mixed model of SAS. The model considered the treatments (P4-W vs. Con), the time, as well as the interaction between treatments and time as fixed effects, and the ewe within each group as a random effect. Post-hoc comparisons were performed with LSD. The area under the curve of cortisol and the frequency of each type of behaviour, excepting eliminations and escape attempts, were compared using ANOVA. The frequency of eliminations and escape attempts, which did not have normal distribution, was compared with the Mann-Whitney test.
The latencies and occurrences of vocalizations and eliminations were analyzed using the survival test. Data are presented as mean±SEM.

RESULTS

Description of the progesterone treatment

Progesterone concentration in P4-profile ewes during P4 treatment and after CIDRs withdrawal is presented in Figure 1. Progesterone concentrations increased on Day 1, remaining high until Day 4, and in luteal concentrations until CIDR withdrawal. P4 returned to basal concentrations 4 h after CIDRs were removed (Figure 1).

Cortisol

Cortisol concentrations of P4-W and Con ewes before the social isolation were 24.3±2.8 and 30.3±4.0 nmol/L, respectively (P = 0.14). Cortisol concentration increased after the social isolation test (P < 0.001) in both groups, reaching maximum concentration at 20 (P < 0.001) min, and did not return to initial concentrations until 60 min after the social isolation test (P < 0.001) (Figure 2). Ewes of the P4-W group presented a greater cortisol concentration than Con ewes (P = 0.04; Figure 2). There was an interaction between time and treatment in the change of cortisol concentration (P = 0.008): the increase in P4-W ewes was greater 0, 10, 20 and 30 min after the social isolation test than in Con ewes (P < 0.02; Figure 2). P4-W ewes also tended to have a greater increase of cortisol than Con ewes 45 min after the social isolation test (P = 0.08). Moreover, P4-W ewes had greater area under the curve for cortisol concentration than Con ewes (41,785%±4,156% vs. 25,682%±4,565% during 75 min; P = 0.02).

Progesterone

As treatments did not affect P4 concentration after the social isolation test, data from both groups are presented pooled (Figure 3). Progesterone concentrations before the social isolation was 1.44±0.15 nmol/L. P4 concentration increased immediately after the social isolation test (P < 0.001) and remained elevated 30 min after the test (P < 0.001).

Body surface temperature

Body surface temperatures on the thorax (P < 0.03) and abdomen (P < 0.03) increased after the social isolation test without significant differences between groups. Data from both groups are presented pooled in Figure 4.
Behavioural recording

The P4-W and Con groups did not present differences in the frequency of any behaviour recorded during the social isolation test (Table 1).

DISCUSSION

This is the first study that demonstrates that pretreatment with P4 increases the physiological stress response to social isolation in ewes. The use of anoestrous ewes, which have very low oestrogen concentrations and do not ovulate after a single P4 treatment such as that applied in this experiment, allows us to isolate the P4 effects from possible effects of oestrogen increases after P4 concentrations decrease. This means that the physiological status of different ewes should be considered when temperament tests are performed, while management systems should also consider physiological status to minimize welfare problems. After social isolation, the changes in cortisol, the main endocrine indicator of an acute stress response in sheep, were greater in P4-W ewes. Although it is known that CRF, ACTH and glucocorticoids have direct stimulating effects on the behavioural response to stressors, we did not observe differences between the groups in the behavioural displays during the social isolation test. One explanation could be that differences in the CRF, ACTH and/or cortisol responses were not strong enough to induce differences in the behavioural response, or alternatively, the neuroendocrine changes were restricted to the adrenal level. Overall, this result provides support to the hypothesis that the decrease in P4 concentrations increases the sensitivity of the hypothalamic-pituitary-adrenal axis to stressors.

Overall, we observed responses in cortisol and body temperature and typical behavioural responses dis-

<table>
<thead>
<tr>
<th>Variable</th>
<th>P4-W</th>
<th>Con</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lines crossed (n)</td>
<td>56.3 ± 6.7</td>
<td>53.4 ± 4.4</td>
</tr>
<tr>
<td>Periphery lines crossed (n)</td>
<td>36.8 ± 4.1</td>
<td>41.1 ± 2.9</td>
</tr>
<tr>
<td>Sniffing (n)</td>
<td>20.9 ± 3.0</td>
<td>23.1 ± 3.6</td>
</tr>
<tr>
<td>Escape attempts (n)</td>
<td>0.2 ± 0.1</td>
<td>2.4 ± 2.2</td>
</tr>
<tr>
<td>Freezing (n)</td>
<td>31.9 ± 4.3</td>
<td>32.1 ± 2.5</td>
</tr>
<tr>
<td>Eliminations events (n)</td>
<td>0.7 ± 0.1</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Vocalizations (n)</td>
<td>27.4 ± 6.6</td>
<td>31.7 ± 7.6</td>
</tr>
<tr>
<td>Time in periphery squares (min)</td>
<td>7.2 ± 0.6</td>
<td>7.9 ± 0.5</td>
</tr>
<tr>
<td>Latency to first elimination (min)</td>
<td>4.2 ± 1.5</td>
<td>6.9 ± 1.4</td>
</tr>
<tr>
<td>Latency to first vocalization (min)</td>
<td>2.5 ± 1.3</td>
<td>3.3 ± 1.2</td>
</tr>
</tbody>
</table>

Figure 3. Progesterone changes in ewes before and after social isolation test. BSIT: progesterone concentration 5 min before the social isolation test; ASIT: progesterone concentration immediately after the social isolation test. Shaded area shows the period of the social isolation test. Different letters indicate significant differences over time ($P<0.03$).

Figure 4. Body surface temperature on the thorax (▲) and abdomen (▲) of ewes after the social isolation test. BSIT: temperature values 5 min before social isolation test; ASIT: temperature values immediately after social isolation test. Shaded area shows the period of the social isolation test. Different letters indicate significant differences over time ($P<0.03$).
played by ewes exposed to a social isolation test. We also observed an increase of P4 concentrations after social isolation. As it has been previously reported that the administration of ACTH induces a P4 response of adrenal origin, P4 changes may also be considered a reliable indicator of a stress response. An increase in body surface temperature was also a component of the stress response. Although it is widely known that rectal temperature increases during a stress response, the use of body surface temperature has the advantage of being non-invasive and fast to measure; as the animals need to be immobilized for a shorter period, this may reduce the stress caused by the procedure. Our results provide support to include these two variables in future studies of stress response in sheep.

When the experiment was designed there were no data in the literature showing the pattern of P4 reduction after a CIDR treatment in anoestrous ewes. Moreover, there was no information on the changes of sensitivity to stressors according to the different time lengths after the P4 treatment ended. The length of P4 treatment or the interval between P4 treatment ended and the application of the social isolation test may have limited the responses of the ewes. We tested the ewes 24 h after the CIDR withdrawal based on the assumption that P4 concentrations would have returned to basal concentrations shortly before that. However, P4 concentrations had returned to basal levels approximately 20 h earlier, just 4 h after CIDR withdrawal, which could mean that the period of maximum sensitivity to stressors might have ended some hours earlier. Another consideration should be the length of treatment, as maximum P4 concentrations were achieved earlier and decreased slowly until the moment of CIDR withdrawal. Corroborating this, in a later study we observed an increase in reactivity of female calves to humans 16 h after CIDRs withdrawal following 5 d of treatment (Magri G, Freitas-de-Melo A, Ungerfeld R, unpublished results). It may therefore be interesting to test whether the responses are greater if the CIDRs are removed earlier when P4 values are maximum (e.g., Day 4 of the treatment). Thus, a shorter CIDR treatment combined with the application of the stressor earlier after P4 concentrations reduction might result in greater behavioural and physiological responses.

CONCLUSIONS

P4 pretreatment appears to augment the stress response to social isolation in anoestrous ewes without differences in the behavioural response.

ACKNOWLEDGEMENTS

We would like to thank Florencia Baracochea, Gabriela Magri, Laura Morena, Magdalena Cassarino, Marcela Canabal for their assistance with data collection, and Damián González and Alberto García for their help with animal management.

FUNDING

Financial support was provided by PEDECIBA (Uruguay) and INIA. MJ Hötzel was supported by CNPq, Brazil.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

REFERENCES

Prevalence and determinants of type 2 diabetes mellitus in a Greek adult population

Sofia Tsirona, Fotis Katsaros, Alexandra Bargiota, Stergios A. Polyzos, George Arapoglou, George N. Koukoulis

Department of Endocrinology and Metabolic Diseases, University Hospital, School of Medicine, University of Thessaly, Biopolis, Larissa, Greece

ABSTRACT

The prevalence of diabetes mellitus (DM) is increasing worldwide reaching epidemic proportions. The aim of the present study was to estimate the prevalence of DM in Thessaly, a large region of Central Greece, and to extrapolate our results to the population of the entire country. A random sample of 805 adults (421 females and 384 men) living in Thessaly, aged 18-80 years, was surveyed. After completing a questionnaire about health status and a thorough physical examination, a blood sample was obtained from each participant for biochemical analysis. Participants with fasting glucose levels between 100-125 mg/dl underwent an oral glucose tolerance test (OGTT). A second survey was also conducted, via telephone call-interviews, in a randomly selected sample age- and sex-stratified to the country’s adult population in order to extrapolate the DM data from Thessaly to the whole country. The frequency of DM based on patient history and fasting blood glucose levels was 6.96%, comparable to that observed in the telephone-based nationwide survey (7.38%, p=0.669). However, after the OGTT an additional 3.72% of the population had undiagnosed DM, increasing DM prevalence to 10.68% (age adjusted 11.77%). The prevalence of pre-diabetes was 8.70%, with impaired fasting glucose at 5.84% and impaired glucose tolerance at 2.86%. The prevalence of DM was significantly higher in men (14.58%) than in women (7.13%, p<0.001), increased with age in both sexes and was more prevalent in hypertensive (p<0.001) and obese subjects (p=0.001) and in those living in rural areas (p=0.003). In the multiple logistic regression analysis, significant predictors of pre-diabetes and DM together were age, homeostasis model of assessment of insulin resistance (HOMA-IR), alcohol consumption and educational status, whereas those of DM alone were age, HOMA-IR and triglycerides. Extrapolating our data to the whole country, the age-adjusted prevalence of DM was estimated at 11.97% (men 13.98%, women 9.25%), clearly indicating a major public health problem.

Key words: Diabetes mellitus, Frequency, Greece, OGTT, Prevalence, Thessaly
INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by a constellation of abnormalities of glucose homeostasis, which are associated with significant acute and chronic complications. It constitutes a global health problem, as the number of people with DM is increasing worldwide, this due to population growth, aging, urbanization, lifestyle modifications and the increasing prevalence of obesity and physical inactivity. Currently, DM has reached epidemic proportions; the prevalence of DM worldwide was 2.8% in the year 2000, 6.4% in 2010 and is predicted to reach 7.7% by 2030, while the prevalence of impaired glucose tolerance (IGT) was 7.9% in 2010 and is predicted to be 8.4% by 2030. IGT is a serious condition, considering that pre-diabetic individuals may develop type 2 DM in the subsequent 10 years and are also at higher risk for cardiovascular diseases than normoglycemic individuals. 

Data from different studies indicate that the prevalence of DM varies widely worldwide among populations due to differences in genetic susceptibility and environmental factors. In Greece, only a limited number of population-based studies have been conducted on the prevalence of DM. Most of these studies concern small areas, either rural or urban, are based on self-reported data and are retrospective or pertain to non-randomly selected populations.

The aim of this study was to estimate the prevalence of DM and IGT in the population of Thessaly, a large region of Central Greece. Secondary aims of the study were: a) to determine factors related to DM and b) to extrapolate the results to the entire Greek population using the self-reported data from a nationwide survey.

SUBJECTS AND METHODS

The study was carried out in accordance with the Declaration of Helsinki. Written informed consent was signed by all the participants and the study protocol was reviewed and approved by the scientific and ethics committee of the University Hospital of Larissa.

Sample selection

The sample of the study consisted of 805 individuals, 421 females and 384 males, who were drawn from

the Adult Regional Greek Obesity Survey (ARGOS), a community-based health and nutrition study. This survey was carried out in the region of Thessaly, Central Greece, with a population of approximately 625,000 adult inhabitants, aged 18-80 years, living in urban (60%) or rural (40%) areas for at least one year. The details of the random sample selection were described in our previous study. Briefly, the sample was randomly selected from the capital city of each of the four provinces of Thessaly, as well as from other towns and villages, in proportion to their population. The baseline evaluation included: demographic characteristics, age, gender, profession, personal and family history of DM, hypertension, hypercholesterolemia, smoking, dietary and alcohol consumption habits. According to the population census, areas with less than 2,000 inhabitants were defined as rural. The data were collected from 2003 to 2005.

In order to extrapolate these data to the prevalence of DM throughout the country, we conducted a second survey in a randomly selected sample, age- and sex-stratified to the country’s adult population according to the census of 2001 of the National Service for Statistics. The communication was performed via telephone call-interviews and was selected by a List-Assisted Random Digit Dialing (RDD) sampling method. Interviews were performed with 10,000 individuals (aged 18-80 years). The sample size for the whole country’s population survey was estimated according to power analysis accepting a confidence level of 99%, a margin of error of 1.5% and a response distribution of 50%. From the 10,000 adults reached by telephone, 7,238 agreed to participate in the study (refusal rate 28%).

Each subject was asked whether he/she had any problem with their blood glucose level. In the subgroup of individuals who responded that they had DM, a second telephone-based interview was conducted which included questions about demographic and socioeconomic characteristics, duration of DM, physical activity, kind of treatment, etc. This study was conducted from 24/9/2008 to 2/10/2008.

Procedure

The participants from the region of Thessaly were asked to come to the nearest public health center in the morning (8.30-9.30 am) after having fasted for
at least 12 hours. Subjects were asked to bring all of their medications.

Each subject underwent a thorough physical examination. Body weight and height, waist and hip circumference and blood pressure were measured as previously described. A blood sample was obtained from all the participants for the measurement of glucose, insulin, total cholesterol, high density lipoprotein cholesterol (HDL-C) and triglycerides.

The blood samples were centrifuged on site and the serum and plasma specimens were transferred immediately, in cold boxes filled with ice, to the university hospital laboratories and stored at -70°C until the measurement. In addition, each participant was asked by the doctor about his/her health status and health behaviors (smoking, alcohol intake, dietary habits, etc.).

Subjects with fasting plasma glucose (FPG) levels ≥126mg/dl in two consequent measurements were considered as diabetics and those with FPG levels <100mg/dl normoglycemic. Participants with FPG levels between 100 and 125 mg/dl were considered to have impaired fasting glucose (IFG) and 64 out of 100 subjects with IFG underwent an oral glucose tolerance test (OGTT) after 3 days of ad libitum carbohydrate diet. Subjects were classified as being diabetics, with IGT or IFG, when the 2-hour plasma glucose level was ≥200mg/dL, ≥140 but <200mg/dL or <140mg/dl, respectively, according to the WHO criteria. Pre-diabetes is a term used to describe a high-risk state for the development of diabetes. It is assumed that pre-diabetes is the combination of IFG and IGT. For the quantification of insulin resistance, the homeostasis model of assessment of insulin resistance (HOMA-IR) was calculated. Fasting total cholesterol levels ≥200mg/dl, triglycerides levels ≥150mg/dl and HDL-C levels <40mg/dl for men and <50mg/dl for women were respectively defined as hypercholesterolemia, hypertriglyceridemia and low HDL cholesterol according to the National Cholesterol Educational Program Adult Treatment Panel III guidelines. Participants using lipid lowering agents were considered as having hyperlipidemia.

Body mass index (BMI; kg/m²) was calculated from the body weight and height according to Quetelet’s formula: BMI= weight (kg)/height² (m²). Waist circumference (WC) and the calculated waist-to-hip ratio (WHR) were used as estimates of central obesity. Abdominal obesity was defined by a WC greater than 102cm for men and greater than 88cm for women. Weight status was classified according to the WHO definitions: underweight (UW): BMI <18.5, normal weight (NW): BMI 18.5 - 24.9, overweight (OW): BMI 25 - 29.9, obese: BMI ≥30 and extremely or morbidly obese (MO): BMI ≥40. Hypertension was defined as blood pressure ≥140 or ≥90mmHg for systolic or diastolic blood pressure, respectively, or the use of antihypertensive medication. Those who had smoked for over a year and those who had stopped smoking during the last year were defined as smokers. The rest were non-smokers.

**Laboratory analysis**

Plasma insulin levels were measured using a commercially available immunoradiometric assay (IRMA) kit (Immunotech Beckman Counter Company, Marseille, France, PW). The assay’s sensitivity for insulin measurements was 0.51 IU/mL and the interassay coefficient of variation (CV) 3.4% without any cross-reactivity with proinsulin. Total cholesterol, HDL-C, triglycerides and glucose were measured by standard methods on an automatic analyzer (Olympus 600; Medicon, Athens, Greece). Specifically regarding plasma glucose, this was measured by an automated enzymatic assay (hexokinase). LDL-C was calculated according to the Friedwald equation.

**STATISTICAL ANALYSIS**

Results are presented as mean ± standard deviation (SD) for normally distributed continuous variables, as median (range) for non-normally distributed continuous variables and as frequencies for qualitative variables. The Kolmogorov-Smirnov test was used to check the normality of distributions of the continuous variables. The independent sample Student’s t-test or the Mann-Whitney U-test was used to compare between group differences for continuous variables when there were two groups. The chi-square test or Fisher’s exact test was used to compare between group differences for qualitative variables. The Cochran-Armitage test was also used to explore whether there was a clear trend of DM rate with age and BMI, treated as categorical variables. One-way ANOVA, with Bonferroni cor-
Diabetes prevalence in Greece

Correction for multiple pairwise comparisons, was used to compare between group differences for continuous variables when there were more than two groups. The age-adjusted prevalence of DM, IFG, IGT and prediabetes was calculated by the direct method. The mid-year (2007 and 2008) population estimates by sex and age groups was used as a “standard” population.17

Multiple logistic regression analysis (“stepwise” method) was performed to identify variables independently associated with DM. All variables that provided p-value <0.01 in univariate analysis were entered in each model of multiple regression analysis. Variables that were not normally distributed were logarithmically transformed for the need of these analyses. A two-sided p-value <0.05 was considered statistically significant in all the above tests. Regression analyses were performed with SPSS 21.0 for Macintosh (IBM Corp., Armonk, NY, USA).

RESULTS

A total of 805 individuals (384 (47.7%) men and 421 (52.3%) women), aged 47.0±13.3, were included in the study. According to BMI, 29.3% had normal weight, 40.5% were overweight and 30.2% were obese (men: 17.7%, 51.1%, and 31.2%; women: 39.9%, 30.9%, and 29.2%, respectively). Abdominal obesity was present in 42.5% (39.1% of men and 45.6% of women).

The prevalence of DM, based on patient history and fasting plasma glucose levels, was 6.96%, without any significant difference between males and females (Table 1). 12.42% of individuals had IFG, with significantly higher rates in men compared to women (Table 1). In individuals with IFG who underwent an OGTT, we found that 29.70% of them were diabetics, 23.40% had IGT and the remaining 46.90% had IFG (Table 2). Similar rates of undiagnosed DM were observed in men and women, despite a non-significant trend towards higher rates in men. After extrapolating the frequency of DM, IGT and IFG to the whole number of subjects with fasting plasma glucose 100-125mg/dl, it was found that an additional 3.72% of subjects had undiagnosed DM, increasing the overall prevalence of DM in our sample population to 10.68% with significantly higher frequency in men compared to women (14.58% vs. 7.13%, p<0.001) (Table 3). The overall prevalence of pre-diabetes was 8.70% (IFG 5.84% and IGT 2.86%), with significantly higher frequency of IFG and pre-diabetes in males compared to females (Table 3).

In the group of 7,238 subjects interviewed by telephone, known DM was declared by 531 (7.38%),

---

Table 1. Prevalence of normoglycemia (NG), impaired fasting glucose (IFG) and diabetes mellitus (DM) based on fasting plasma glucose levels

<table>
<thead>
<tr>
<th>Plasma glucose levels (mg/dl)</th>
<th>Males</th>
<th></th>
<th>Females</th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>&lt;100 (NG)</td>
<td>280/384</td>
<td>72.92</td>
<td>369/421</td>
<td>87.65*</td>
<td>649/805</td>
</tr>
<tr>
<td>100-125 (IFG)</td>
<td>72/384</td>
<td>18.75</td>
<td>28/421</td>
<td>6.65*</td>
<td>100/805</td>
</tr>
<tr>
<td>≥126 (DM)</td>
<td>32/384</td>
<td>8.33</td>
<td>24/421</td>
<td>5.70</td>
<td>56/805</td>
</tr>
</tbody>
</table>

*p<0.001 compared to males

Table 2. The frequency distribution of IFG, IGT and DM in the 64 subjects who underwent OGTT

<table>
<thead>
<tr>
<th>Plasma glucose levels (mg/dl)</th>
<th>Males</th>
<th></th>
<th>Females</th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>2 hours after the 75 gr glucose load</td>
<td>46</td>
<td></td>
<td>18</td>
<td></td>
<td>64</td>
</tr>
<tr>
<td>&lt;140 (IFG)</td>
<td>21</td>
<td>45.65</td>
<td>9</td>
<td>50.00</td>
<td>30</td>
</tr>
<tr>
<td>140-199 (IGT)</td>
<td>10</td>
<td>21.74</td>
<td>5</td>
<td>27.78</td>
<td>15</td>
</tr>
<tr>
<td>≥200 (DM)</td>
<td>15</td>
<td>32.61</td>
<td>4</td>
<td>22.22</td>
<td>19</td>
</tr>
</tbody>
</table>

indicating a prevalence comparable to that found in Thessaly (p=0.669). After adjustment for age, the prevalence of DM in Thessaly was found to be 11.77%, whereas after extrapolating these data to the whole country, the age-standardized prevalence of DM was calculated at 11.97% (Table 4).

The subsequent analyses included 769 subjects after exclusion of those who declined to undergo the OGTT. The prevalence of DM was found to increase significantly with age (p=0.001) in both sexes, reaching its highest level in the group of 70-80 year-olds without any significant difference between men and women (Figure 1, Table 5). Rural residents had a significantly higher prevalence of DM than urban residents; when it was examined separately, women living in rural regions had significantly higher rates of DM, whereas statistical significance was marginally lost among men. However, this difference was no longer significant after controlling for age. The prevalence of DM was significantly higher in married than in single individuals, but it lost statistical significance when tested separately in men and women (Table 5).

Comparative data regarding cardiovascular risk factors are presented in Table 6. As expected, men and women with higher BMI or abdominal obesity had a significantly higher prevalence of DM. On the other hand, subjects with DM or IGT had significantly higher BMI and WC compared to individuals with normoglycemia (31.51±6.64 vs. 27.72±5.24 kg/m², p=0.001; 106.57±13.15 vs 93.37±14.79 cm, p=0.001 and 30.91±5.75 vs 27.72±5.24 kg/m², p=0.006; 101.03±12.57 vs 93.37±14.79 cm, p=0.003, respectively). An association was also found between DM and hypertension. Men and women with hypertension had a significantly higher prevalence of DM compared to individuals without hypertension (Table 6). Smoking was also negatively associated with DM rates. Smokers had lower rates of DM compared to non-smokers. Hypercholesterolemia was not associated with DM prevalence, nor were hypertriglyceridemia and low HDL (data not shown).

In multiple logistic regression analysis, age, HOMA-IR, alcohol consumption and educational status were independently associated with the presence of T2DM/pre-diabetes (IFG and IGT) when compared...
Table 5. The prevalence of diabetes mellitus among men and women based on sociodemographic characteristics

<table>
<thead>
<tr>
<th>Males</th>
<th>Females</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>Age (years) (n=769)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18-29</td>
<td>0/26</td>
<td>0.00</td>
</tr>
<tr>
<td>30-39</td>
<td>2/59</td>
<td>3.39</td>
</tr>
<tr>
<td>40-49</td>
<td>12/110</td>
<td>10.91</td>
</tr>
<tr>
<td>50-59</td>
<td>12/78</td>
<td>15.38</td>
</tr>
<tr>
<td>60-69</td>
<td>12/64</td>
<td>18.75</td>
</tr>
<tr>
<td>70-80</td>
<td>9/21</td>
<td>42.86</td>
</tr>
<tr>
<td>p value</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>p value (Cochran-Armitage test)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Marital status (n=741)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>3/50</td>
<td>6.00</td>
</tr>
<tr>
<td>Married</td>
<td>41/299</td>
<td>13.71</td>
</tr>
<tr>
<td>p value</td>
<td>0.167</td>
<td>0.227</td>
</tr>
<tr>
<td>Education (n=769)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>30/154</td>
<td>19.48</td>
</tr>
<tr>
<td>Secondary</td>
<td>15/136</td>
<td>11.03</td>
</tr>
<tr>
<td>Higher</td>
<td>2/68</td>
<td>2.94</td>
</tr>
<tr>
<td>p value</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Table 6. Prevalence of diabetes mellitus among men and women based on cardiovascular risk factors</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>BMI (kg/m²) (n=769)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI &lt;25</td>
<td>3/62</td>
<td>4.84</td>
</tr>
<tr>
<td>BMI 25-29.9</td>
<td>21/184</td>
<td>11.41</td>
</tr>
<tr>
<td>BMI ≥30</td>
<td>23/112</td>
<td>20.53</td>
</tr>
<tr>
<td>p value</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>p value (Cochran-Armitage test)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Abdominal obesity (n=769)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>29/154</td>
<td>18.83</td>
</tr>
<tr>
<td>No</td>
<td>18/204</td>
<td>8.82</td>
</tr>
<tr>
<td>p value</td>
<td>0.007</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypertension (mmHg) (n=769)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>28/121</td>
<td>23.14</td>
</tr>
<tr>
<td>No</td>
<td>19/237</td>
<td>8</td>
</tr>
<tr>
<td>p value</td>
<td>0.001</td>
<td>0.006</td>
</tr>
<tr>
<td>Smoking (n=769)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>17/180</td>
<td>9.44</td>
</tr>
<tr>
<td>No</td>
<td>30/178</td>
<td>16.85</td>
</tr>
<tr>
<td>p value</td>
<td>0.042</td>
<td>0.023</td>
</tr>
<tr>
<td>Hypercholesterolemia (n=769)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>35/254</td>
<td>13.78</td>
</tr>
<tr>
<td>No</td>
<td>12/104</td>
<td>11.54</td>
</tr>
<tr>
<td>p value</td>
<td>0.610</td>
<td>&lt;0.999</td>
</tr>
</tbody>
</table>
with non-diabetic individuals (Table 7); individuals of higher age, HOMA-IR and alcohol consumption, but lower educational status were at higher risk for T2DM. These results remained essentially unchanged when wine consumption replaced alcohol consumption in this regression model.

Age, HOMA-IR and serum triglycerides were independently associated with the presence of T2DM when pre-diabetes (IFG and IGT) was excluded from the analysis (Table 8); individuals of higher age, HOMA-IR and triglycerides were at higher risk for T2DM.

**DISCUSSION**

The present study assessed the prevalence of DM in a random sample of adults living in a large region of Central Greece with both urban and rural areas. We found that a large proportion of adults (10.68%; men 14.58% and women 7.13%) had DM, while a smaller proportion (2.86%) had IGT and 8.70% had pre-diabetes. It should be emphasized that DM was undiagnosed in 34.88% of cases.

Epidemiological data regarding the prevalence of DM are rare in Greece. The first epidemiological studies concerning its prevalence, using glycosuria as a screening method, were conducted in the early 70’s showing low rates of DM. Twenty years later, similar estimates (1.52%) were found by Lionis et al in a retrospective study based on reviewing all medical records of a small rural district health care center and performing OGTT in subjects with non-diagnostic values of fasting glucose, based on the WHO criteria. Our estimated prevalence of DM is in accordance with that of a study synchronous to our own conducted in the urban area of Attica (DM prevalence 6.74%), in subjects free of apparent cardiovascular diseases, indicating a potential selection bias. Compared to another synchronous study conducted in a rural area, including individuals aged 1-99 years, our DM prevalence is higher (10.68% vs. 7.40%). However, when we restricted their age-range to 20-80 years, the results concerning DM prevalence were comparable to our data in the rural area (9.35% vs. 11.52%). Close to our results is also the prevalence of DM in Cyprus (10.3%; 6.5% with known DM and 3.8% with undiagnosed DM) with population characteristics comparable to those of Greece.

The prevalence of DM seems to be on the increase worldwide, although different racial, cultural, financial and habitual factors may influence its frequency across

<table>
<thead>
<tr>
<th>Covariate</th>
<th>B coefficient</th>
<th>Adjusted Odds Ratio</th>
<th>p-value</th>
<th>95% CI for adjusted odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>0.054</td>
<td>1.056</td>
<td>0.001</td>
<td>1.022 – 1.090</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.293</td>
<td>1.340</td>
<td>&lt;0.001</td>
<td>1.216 – 1.477</td>
</tr>
<tr>
<td>Alcohol consumption (Y/N)</td>
<td>0.980</td>
<td>2.664</td>
<td>0.002</td>
<td>1.415 – 5.017</td>
</tr>
<tr>
<td>Educational status (Lower vs Higher)</td>
<td>-0.591</td>
<td>0.554</td>
<td>0.029</td>
<td>0.326 – 0.942</td>
</tr>
</tbody>
</table>

Individuals without T2DM or IFG or IGT were rated as 0 and those with T2DM or IFG or IGT as 1 within the dependent variable.

<table>
<thead>
<tr>
<th>Covariate</th>
<th>B coefficient</th>
<th>Adjusted odds ratio</th>
<th>p-value</th>
<th>95% CI for adjusted odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>0.082</td>
<td>1.086</td>
<td>&lt;0.001</td>
<td>1.044 – 1.129</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.258</td>
<td>1.294</td>
<td>&lt;0.001</td>
<td>1.161 – 1.442</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>0.004</td>
<td>1.004</td>
<td>0.020</td>
<td>1.001 – 1.007</td>
</tr>
</tbody>
</table>

Individuals without T2DM or IFG or IGT were rated as 0 and those with T2DM as 1 within the dependent variable.
the world. In Europe, the known comparative age-standardized prevalence of DM in 2010 ranged from 2.1% to 12%, according to the International Diabetes Federation. Using only FPG for the diagnosis, the overall prevalence of DM in Greece (6.96%) appears to be lower than that documented recently in the USA in adults over 20 years old (9.3%). Undiagnosed DM has been reported to carry a similar risk of mortality to that of diagnosed DM. However, the estimated prevalence depends on the method used. The WHO 1999 protocol is considered the gold standard for estimating undiagnosed DM, using measurements of FPG and 2-hour glucose. According to the 2013 IDF Diabetes Atlas, globally, undiagnosed DM is estimated to account for 45.8% of all cases, with considerable variability across different world regions ranging from 24.5% to 75.1%. This variability is attributed to a number of determinants, among which income is an important one affecting health care and the educational level of citizens. Health systems are generally more developed in high-income countries and thus DM is likely to be detected earlier. However, even within each country, access to health care may be lower in rural compared to urban areas. The proportion of undiagnosed DM ranges in Europe from 30%, based on FPG, to 44-61%, based on OGTT, in two regional and four nationwide studies. Compared to data found in the well-controlled European studies concerning the prevalence of undiagnosed DM based on OGTT, our results are lower (approximately 35% of DM), possibly indicating more timely diagnosis of DM in Thessaly. Of course, regional differences in the proportion of undiagnosed DM are likely to affect the total prevalence of DM and may partly explain its variability among different countries with a comparable socioeconomic environment.

The OGTT-based studies provide estimates of pre-diabetes (combined IFG and IGT), which is an important risk factor for DM. Regarding the prevalence of pre-diabetes in Greece, to the best of our knowledge, there is only one previously mentioned survey in a rural region, conducted in 2002. In the age-comparable group of the above study, the prevalence of pre-diabetes was 4.22%, this being similar to our results when we compared the group of participants living in rural areas (5.56%). Our results regarding the prevalence of pre-diabetes (8.7%) are comparable to those found in Cyprus (9.5%), but lower than those in the abovementioned European countries where prevalence ranges from 14% to 30%, and the results of the NHANES (2005-2008) survey which showed that the pre-diabetes rate among non-Hispanic whites in the USA was 29.5%.

The prevalence of DM in our cohort was found to be significantly higher in men compared to women, the latter being analogous to published data from Spain. It was observed that the prevalence of diabetes was higher in rural areas, but after controlling for age, the statistical significance of this difference was lost, confirming previously reported results from Greece.

Age, education, HOMA-R and alcohol consumption were independently associated with DM/pre-diabetes. When pre-diabetes was excluded from the regression analysis, only age, HOMA-R and triglycerides remained independently associated with DM. Given that this was an observational study, a causal relationship cannot be established; however, we could speculate that alcohol consumption may influence factors that contribute to progression of metabolic disturbances (e.g., inflammation, oxidative stress and/or beta-cell dysfunction). Our results are in accordance with those found in a recent meta-analysis showing that alcohol consumption of more than 50 gr/day increases the risk for DM. Other risk factors such as obesity and sedentary life associated with alcohol consumption may also explain our results.

Age is an independent risk factor for DM as observed in our series, this concurring with published data from developed countries. Therefore, the expected increase in life expectancy represents a factor contributing to the worldwide increase of DM.

Lower educational status was also found to be associated with DM in our study. This inverse association is in agreement with published data from developed countries. It should also be stressed that a lower educational level is usually associated with low income.

As expected, HOMA-IR, an index of insulin resistance, was independently associated with DM. Obesity is a major negative determinant of insulin sensitivity, as has been shown in human and animal studies, since weight gain correlates positively with insulin resist-
Insulin resistance is a key component of DM, driving beta-cells to a compensatory enhancement of insulin secretion until the time they can no longer produce sufficient insulin, resulting initially in IGT and eventually in type 2 DM. In agreement with published data, we found that cardiovascular risk factors, such as obesity, abdominal obesity and hypertension, are more common in diabetic than non-diabetic individuals. Obesity is an established risk factor for DM. This study showed that the frequency of DM in obese subjects was four times higher compared to individuals with normal weight. This association was also evident among individuals from rural areas, where obesity is significantly higher compared to urban areas.

DM prevalence was lower in smokers than non-smokers. Other studies have also shown that BMI in smokers is lower than in non-smokers, indicating that smokers tend to be thinner, this possibly explaining the inverse association of smoking with DM. Furthermore, since this was an observational study, lower rates of DM in smokers may simply indicate that some ex-smokers had stopped smoking after the diagnosis of DM. On the other hand, smoking may play a role in the development of DM, as this is indicated in a recent study showing that smoking cessation in type 2 diabetics was associated with amelioration of their metabolic parameters. Moreover, smoking is often associated with other unhealthy behaviors such as lack of physical activity, unhealthy diet and high alcohol consumption, factors that favor weight gain and consequently increase the risk of DM.

Our findings are particularly alarming taking into account that DM is associated with increased incidence of long-term microvascular and macrovascular complications that will inevitably result in higher mortality, as this is indicated in the INTERHEART case-control study in Europians.

The present study has certain limitations. First, this was an observational study, therefore no cause-effect relationship could be established. Second, the region of Thessaly may not accurately represent the whole country, although the prevalence of diagnosed DM in our telephone-conducted survey was not different; thus the extrapolation of our data to the whole country should be accepted with some reservation. Moreover, the extrapolation of data of 64 subjects subjected to OGTT to the 100 subjects with IFG may have slightly influenced our results. Finally, we did not measure HbA1c due to limited resources; this might have underestimated the prevalence of DM.

In conclusion, the prevalence of DM and prediabetes in Thessaly seems to be comparable to that observed in other European countries. This should be seriously considered by the Greek Health Agency, since DM-associated complications lead to increased cost, morbidity and mortality. Screening programs may be of importance so as to earlier diagnose individuals who are unaware of being diabetics and thereby more efficiently follow up those with pre-diabetes.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest and that they received no specific funding for this article.

REFERENCES

17. Concise statistical yearbook 2009, Hellenic statistical authority, Piraeus, Greece, 2010
41. Kumari M, Head J, Marmot M, 2004 Prospective study
The relationship between retinol-binding protein 4 and apolipoprotein B-containing lipoproteins is attenuated in patients with very high serum triglycerides: A pilot study

Georgios A. Christou,1 Constantinos C. Tellis,2 Moses S. Elisaf,3 Alexandros D. Tselepis,2 Dimitrios N. Kiortsis1

1Laboratory of Physiology, Medical School, 2Laboratory of Biochemistry, Department of Chemistry, 3Department of Internal Medicine, Medical School, University of Ioannina, Ioannina, Greece

ABSTRACT

OBJECTIVE: The investigation of the association between retinol-binding protein 4 (RBP4) and lipoproteins in subjects with hypertriglyceridemia. DESIGN: Forty-six obese or overweight hypertriglyceridemic patients were studied at baseline and 20 of them underwent a hypocaloric low-fat diet for 3 months. RESULTS: Plasma RBP4 levels were positively correlated with serum triglycerides (TG) in the subgroup of patients with TG <200 mg/dL (r=0.453, p=0.039) and negatively correlated with TG in patients with TG ≥200 mg/dL (r=-0.487, p=0.019). In the subgroup with TG <200 mg/dL, subjects with circulating RBP4 above the median 46 mg/L had higher levels of intermediate density lipoprotein-cholesterol (IDL-C), low-density lipoprotein-cholesterol (LDL-C) and apolipoprotein B (ApoB), while these differences were absent in patients with TG ≥200 mg/dL. The associations of percentage changes of circulating RBP4 with the percentage changes of LDL-C, very low-density lipoprotein-cholesterol (VLDL-C) and ApoB were positive after the first month and 3 months of diet for patients with baseline TG <200 mg/dL, while no correlations existed for patients with TG ≥200 mg/dL. CONCLUSIONS: The positive association between circulating RBP4 and ApoB-containing lipoproteins in a steady metabolic state, as well as during a hypocaloric diet, appears to be attenuated in patients with very high TG.

Key words: Apolipoprotein B, Diet, Obesity, Retinol-binding protein 4, Triglycerides

INTRODUCTION

Retinol-binding protein 4 (RBP4), a transport protein for vitamin A, is synthesized mainly by the hepatocytes and secondly by the adipose tissue.1 Plasma RBP4 levels are upregulated in insulin resistant states associated with obesity, while RBP4 also induces insulin resistance.1,2 Furthermore, elevated circulating RBP4 has been associated with the development of cardiovascular disease.3–6 Circulating RBP4 has been shown to be positively correlated with serum triglycerides (TG)
and low-density lipoprotein-cholesterol (LDL-C) and negatively with high-density lipoprotein-cholesterol (HDL-C). Among these associations the strongest and the most consistently reported has been the association with TG. Moreover, serum RBP4 levels have been shown to increase TG in mice. These data indicate that RBP4 are possibly associated with TG metabolism. In the present study we investigated the association of RBP4 with various lipid parameters in subjects with obesity-related hypertriglyceridemia at baseline and during dietary intervention.

MATERIALS AND METHODS

Subjects

In the present study 46 subjects were recruited. They attended the obesity outpatient clinic of the University of Ioannina, Greece. Inclusion criteria were: body mass index (BMI) ≥27 Kg/m² and hypertriglyceridemia (TG ≥150 mg/dL). Exclusion criteria were: age less than 18 years old, pregnancy, breastfeeding, kidney disease, liver disease, gastrointestinal disease, malignancy, any endocrine disorder or metabolic disease other than obesity or type 2 diabetes mellitus (T2DM), alteration of body weight (BW) by up to 5% of the initial BW during the last 3 months, any state of stress or systemic inflammation, taking any of the following drugs within 3 weeks before the start of the study: hypolipidemic agents, antidiabetics, drugs for weight loss, β-blockers or thiazides. Diagnosis of T2DM was reasonably excluded by asking medical history and assessing the values of fasting serum glucose and HbA1c.

Among the total population, 20 participants underwent a hypocaloric low-fat diet for 3 months. A dietitian, taking into account each patient’s basal energy requirements and on an estimation of the subject’s typical activity level, prescribed an individualized low-fat diet promoting a 500 to 1000 kcal reduction in daily energy intake. The administered diets consisted of a mean of 1471±382 kcal/day (ranging from 1085 to 2000 kcal/day depending on the initial BW). The daily distribution of nutrients during the study was as follows: carbohydrates 52.4±3.5%, fat 27.8±2.6% (monounsaturated 15.4±1.7%, polyunsaturated 7.3±1.2% and saturated fatty acids 5.1±1.0%) and protein 19.8±1.2%. There were no differences in diet composition between the study groups. At the end of the 3-month period, the patients were consuming significantly less carbohydrates and saturated fatty acids as well as more monounsaturated fatty acids and n-3 polysaturated fatty acids compared with their baseline diet. All patients were asked to attend the clinic monthly during the treatment in order to assess diet compliance.

Anthropometric measurements and collection of venous blood samples, after an overnight fast of at least 12 h, were performed at baseline, after 1 month and after 3 months of treatment. Plasma samples were stored at -80 °C until analysis.

Measurement of RBP4

Plasma RBP4 was analyzed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (ALPCO DIAGNOSTICS), following the manufacturer’s instructions, as previously described. LDL subclass analysis

LDL subclass analysis was performed electrophoretically by use of high-resolution 3% polyacrylamide gel tubes and the Lipoprint LDL System (Quantimetrix, Redondo Beach, CA), as we have previously described. After electrophoresis, very low-density lipoprotein (VLDL) remained at the origin [retention factor (RF) = 0.0], HDL migrated to the front (RF=1.0). In between, several bands can be detected: MID bands C, B, and A, which correspond mainly to intermediate-density lipoprotein (IDL), as well as up to 7 LDL bands. The LDL-1 and LDL-2 bands correspond to large buoyant LDL particles, whereas bands LDL-3 to LDL-7 correspond to sdLDL particles. A detailed description of the methods used for the measurement of LDL subclasses can be found in an expert consensus document of the “European panel on LDL subclasses”.

Routine biochemical measurements

Total serum cholesterol (TC), HDL-C and TG were measured by enzymatic methods, as previously described. Non-HDL-cholesterol was calculated as TC-HDL-C. Serum apolipoprotein (Apo) A-I and ApoB levels were measured with a Behring Holding GmbH analyzer (Liederbach, Germany).

Serum Creatinine (Cr) levels were determined by
The role of RBP₄ in hypertriglyceridemia

standard laboratory methods. The Modification of Diet in Renal Disease (MDRD) formula was used for the estimation of glomerular filtration rate (eGFR). Body surface area (BSA) was calculated by body weight (BW) and height (H) using the Du Bois formula: BSA = 0.007184 × BW⁰.⁴²⁵ × H⁰.⁷²⁵ (BSA is in m², BW is in kg, and H is in cm).

Statistical analysis

All statistical analyses were performed using the SPSS 16.0 statistical package for Windows (SPSS Inc., 1989-2007). The Kolmogorov-Smirnov test was used to verify the normality of the distributions of the parameters of interest. Normally distributed data were expressed as means±SD. Parameters with skewed distribution were reported as median (range). The paired t-test, independent t-test and Pearson’s correlation analysis were performed for normally distributed parameters, whereas the Mann-Whitney U test and Spearman’s correlation analysis were performed for non-normally distributed parameters. Linear regression analysis was used for the assessment of the relationship between circulating RBP₄ and lipid parameters after adjustment for gender and cGFR. A two-tailed p value <0.05 was considered significant.

RESULTS

Baseline

Participants were 50±14 years old (24 males and 22 females) and their BMI was 36.5±7.3 Kg/m². Plasma RBP₄ levels were higher in males compared with females (54.7±14.2 mg/L vs 42.2±13.6 mg/L, p=0.005). Circulating RBP₄ was positively correlated with Cr (r = 0.367, p = 0.014). Although circulating RBP₄ was not correlated with eGFR, it was negatively correlated with corrected GFR for BSA (cGFR) (r=-0.311, p=0.040).

Plasma RBP₄ levels were not correlated with TG in all patients. Circulating RBP₄ was positively correlated with TG in the subgroup of patients with TG <200 mg/dL (r=0.453, p=0.039) and negatively correlated with TG in the subgroup of patients with TG ≥200 mg/dL (r=−0.487, p=0.019).

Table 1 shows the values of circulating lipoproteins in the subgroups of patients with plasma RBP₄ levels below or above the median 46 mg/L in all patients as well as in subjects with TG < or ≥200 mg/ dL. In all patients, subjects with circulating RBP₄ above the median 46 mg/L had higher levels of IDL-C, LDL-C, nonHDL-C and ApoB and lower levels of ApoE compared with subjects with circulating RBP₄ below 46 mg/L. There was a tendency for higher levels of TC and VLDL-C in subjects with circulating RBP₄ above 46 mg/L.

Dietary treatment

BMI was significantly decreased from baseline (36.2±5.7 Kg/m²) after first month (35.7±4.6 Kg/m², p <0.001), as well as after 3 months of diet (34.9±5.1 Kg/m², p <0.001). Circulating RBP₄ decreased after 3 months of diet (from 51.9±13.8 to 45.7±14.7, p=0.03). Table 2 shows the associations between the percentage change of plasma RBP₄ levels after 1 month of treatment and the percentage changes of IDL-C, LDL-C, VLDL-C, sdLDL-C and ApoB in the total of subjects who underwent dietary treatment and in the subgroups of patients with baseline TG above or below 200 mg/dL. Table 3 shows similar data to Table 2 regarding the 3 months of diet. The associations of percentage changes of circulating RBP₄ with the percentage changes of IDL-C, LDL-C, VLDL-C, sdLDL-C and ApoB were positive over the first month and 3 months of diet for patients with baseline TG <200 mg/dL, while no correlations existed for patients with baseline TG ≥200 mg/dL, except for IDL-C during first month and sdLDL-C for 3 months.
Table 1. The comparison of lipid parameters between subjects with circulating RBP4 below and above the median 46 mg/L, in all patients and in the subgroups with serum triglycerides (TG) < or ≥ 200 mg/dL

<table>
<thead>
<tr>
<th></th>
<th>All patients (n=46)</th>
<th>TG &lt;200 mg/dL (n=22)</th>
<th>TG ≥200 mg/dL (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RBP4&lt;46 mg/L (n=22)</td>
<td>RBP4≥46 mg/L (n=24)</td>
<td>p</td>
</tr>
<tr>
<td></td>
<td>TC (mg/dL)</td>
<td>239 ± 28</td>
<td>259 ± 39</td>
</tr>
<tr>
<td></td>
<td>TG (mg/dL)</td>
<td>235 ± 27</td>
<td>256 ± 26</td>
</tr>
<tr>
<td></td>
<td>HDL-C (mg/dL)</td>
<td>162 ± 18</td>
<td>171 ± 21</td>
</tr>
<tr>
<td></td>
<td>LDL-C (mg/dL)</td>
<td>47 ± 9</td>
<td>44 ± 7</td>
</tr>
<tr>
<td></td>
<td>VLDL-C (mg/dL)</td>
<td>55 ± 10</td>
<td>69 ± 12</td>
</tr>
<tr>
<td></td>
<td>sdLDL-C (mg/dL)</td>
<td>127 ± 14</td>
<td>154 ± 21</td>
</tr>
<tr>
<td></td>
<td>nonHDL-C (mg/dL)</td>
<td>127 ± 14</td>
<td>154 ± 21</td>
</tr>
<tr>
<td></td>
<td>ApoA-I (mg/dL)</td>
<td>141 ± 16</td>
<td>132 ± 15</td>
</tr>
<tr>
<td></td>
<td>ApoB (mg/dL)</td>
<td>141 ± 16</td>
<td>132 ± 15</td>
</tr>
<tr>
<td></td>
<td>ApoE (mg/L)</td>
<td>19 ± 11</td>
<td>14 ± 8</td>
</tr>
<tr>
<td></td>
<td>Lp(a) (mg/dL)</td>
<td>188 ± 21</td>
<td>212 ± 23</td>
</tr>
<tr>
<td></td>
<td>% change of IDL-C</td>
<td>0.296</td>
<td>0.265</td>
</tr>
<tr>
<td></td>
<td>% change of LDL-C</td>
<td>0.290</td>
<td>0.276</td>
</tr>
<tr>
<td></td>
<td>% change of VLDL-C</td>
<td>0.441</td>
<td>0.087</td>
</tr>
<tr>
<td></td>
<td>% change of sdLDL-C</td>
<td>0.447</td>
<td>0.083</td>
</tr>
<tr>
<td></td>
<td>% change of ApoB</td>
<td>0.493</td>
<td>0.045</td>
</tr>
</tbody>
</table>

Data are means ± SD for normally distributed variables or median (range) for non-normal variables.
TC: total cholesterol; TG: triglycerides; HDL-C: high density lipoprotein-cholesterol; IDL-C: intermediate-density lipoprotein-cholesterol; LDL-C: low density lipoprotein-cholesterol; VLDL-C: very low density lipoprotein-cholesterol; sdLDL-C: small dense LDL-cholesterol; nonHDL-C: non-HDL-cholesterol; ApoA-I: apolipoprotein A-I; ApoB: apolipoprotein B; ApoE: apolipoprotein E; Lp(a): lipoprotein(a)
The p value refers to the comparison of the lipid parameters between subjects with serum RBP4 levels below and above 46 mg/L, after performing the independent t-test for normally distributed parameters and Mann–Whitney U test for non-normally distributed parameters.

Table 2. Correlations between percentage change of plasma RBP4 levels over one month of diet and percentage changes of TG, LDL-C, IDL-C, VLDL-C, sdLDL-C and ApoB

<table>
<thead>
<tr>
<th></th>
<th>All patients (n=20)</th>
<th>TG &lt;200 mg/dL (n=10)</th>
<th>TG ≥200 mg/dL (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% change of IDL-C</td>
<td>r 0.296</td>
<td>p 0.265</td>
<td></td>
</tr>
<tr>
<td>% change of LDL-C</td>
<td>r 0.290</td>
<td>p 0.276</td>
<td></td>
</tr>
<tr>
<td>% change of VLDL-C</td>
<td>r 0.441</td>
<td>p 0.087</td>
<td></td>
</tr>
<tr>
<td>% change of sdLDL-C</td>
<td>r 0.447</td>
<td>p 0.083</td>
<td></td>
</tr>
<tr>
<td>% change of ApoB</td>
<td>r 0.493</td>
<td>p 0.045</td>
<td></td>
</tr>
</tbody>
</table>

TG: triglycerides; IDL-C: intermediate-density lipoprotein-cholesterol; LDL-C: low density lipoprotein-cholesterol; VLDL-C: very low density lipoprotein-cholesterol; sdLDL-C: small dense LDL-cholesterol; ApoB: apolipoprotein B.

DISCUSSION

The present study showed that the association between circulating RBP4 and TG was characterized by a biphasic mode, being positive for TG <200 mg/dL and negative for TG ≥200 mg/dL. Moreover, the positive association between RBP4 and ApoB-containing lipoproteins in a steady metabolic state, as well as during the hypocaloric low-fat diet, was found to be attenuated in subjects with TG ≥200 mg/dL.

The relationship between RBP4 and ApoB-containing lipoproteins

The current study demonstrated that circulating RBP4 was positively correlated with serum levels of the ApoB-containing lipoproteins LDL-C, IDL-C and...
Table 3. Correlations between percentage change of plasma RBP4 levels over 3 months of diet and percentage changes of TG, LDL-C, IDL-C, VLDL-C, sdLDL-C and ApoB

<table>
<thead>
<tr>
<th></th>
<th>All patients (n=20)</th>
<th>TG &lt;200 mg/dL (n=10)</th>
<th>TG ≥200 mg/dL (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td>r</td>
</tr>
<tr>
<td>% change of IDL-C</td>
<td>-0.117</td>
<td>0.654</td>
<td>0.783</td>
</tr>
<tr>
<td>% change of LDL-C</td>
<td>0.708</td>
<td>0.001</td>
<td>0.967</td>
</tr>
<tr>
<td>% change of VLDL-C</td>
<td>0.570</td>
<td>0.017</td>
<td>0.760</td>
</tr>
<tr>
<td>% change of sdLDL-C</td>
<td>0.872</td>
<td>&lt;0.001</td>
<td>0.908</td>
</tr>
<tr>
<td>% change of ApoB</td>
<td>0.635</td>
<td>0.005</td>
<td>0.782</td>
</tr>
</tbody>
</table>

TG: triglycerides; IDL-C: intermediate-density lipoprotein-cholesterol; LDL-C: low density lipoprotein-cholesterol; VLDL-C: very low density lipoprotein-cholesterol; sdLDL-C: small dense LDL-cholesterol; ApoB: apolipoprotein B.

VLDL-C. Importantly, this relationship was shown not only at baseline but also during a hypocaloric low-fat diet. Indeed, our study group has demonstrated that circulating RBP4 is possibly associated more consistently and strongly with the metabolism of the ApoB-containing lipoproteins than the metabolism of the ApoA-I-containing lipoprotein HDL.1,10 RBP4 has been found to induce not only the enhancement of hepatic production of ApoB-containing lipoproteins but also the decrease in catabolism of ApoB-containing lipoproteins through the downregulation of the LDL receptor.15 To our knowledge, the association between circulating RBP4 and IDL-C has not previously been investigated.

The relationship between RBP4 and TG in patients with TG <200 mg/dL

The positive association between circulating RBP4 and TG has consistently been reported in studies investigating subjects with variable TG levels, including normal as well as high TG levels.7,8 This relationship is possibly causal since treatment with RNA oligonucleotide against RBP4 was shown to reduce TG levels in mice.9 Vergès et al found that circulating RBP4 in patients with T2DM was negatively correlated with indirect VLDL-apoB fractional catabolic rate (FCR), which represents the VLDL delipidation toward IDL, while there was no significant association with direct VLDL-apoB FCR, which reflects the direct VLDL removal from plasma through receptor-mediated particle uptake.16

The negative association between circulating RBP4 and TG in patients with TG ≥200 mg/dL of the current study was accompanied by the dissociation between circulating RBP4 and ApoB-containing lipoproteins. A possible explanation for these data is the downregulation of plasma RBP4 levels in patients with TG ≥200 mg/dL. Further studies are needed to confirm these findings and elucidate the underlying mechanisms. The dissociation between circulating RBP4 and ApoB-containing lipoproteins in patients with considerable hypertriglyceridemia that was found in the present study implies that the adverse impact of RBP4 on lipoprotein metabolism may be important only in patients without considerable hypertriglyceridemia. In this context, it is prudent to evaluate the effects of RBP4 on lipoprotein metabolism only in patients without considerable hypertriglyceridemia, at least in initial studies.

The negative association between circulating RBP4 and serum ApoE levels in patients with TG ≥200 mg/dL of the current study may be explained by the high serum levels of ApoE, which is carried by remnants of triglyceride-rich lipoproteins in subjects with hypertriglyceridemia.17 The relationship between RBP4 and ApoE has not been investigated in any previous study.

Study strengths and limitations

Strengths of this study include the investigation for the first time of the relationship of RBP4 with lipoproteins in patients who had exclusively hypertriglyceridemia and not in mixed populations with variable TG levels. Secondly, taking into account the many factors that influence circulating RBP4, including renal or liver impairment and drugs affect-
ing metabolism, the present study excluded patients with all these conditions. However, an important limitation of the majority of studies investigating RBP4 was that they did not take into account all these factors. Thirdly, the current study applied a direct measurement of all ApoB-containing lipoproteins through lipoprotein electrophoresis. However, most of the studies investigating the relationship between RBP4 and LDL-C assessed LDL-C by its indirect calculation using the Friedewald equation [LDL-C = TC - (HDL-C + TG/5)], which is less accurate than the direct measurement of LDL-C, especially in subjects with considerable hypertriglyceridemia, as is the case in the present study. Moreover, LDL-C calculated through the Friedewald equation represents a crude estimation of the sum of directly measured LDL-C and IDL-C and thus it is not a highly accurate estimation of true LDL-C.

The results of the present study should be interpreted in light of some limitations. Firstly, the number of patients was not large enough, thus decreasing the statistical power of the study to detect significant associations of circulating RBP4 with serum lipoprotein levels. Therefore, taking into account the small number of study participants, firm conclusions cannot be drawn regarding the relationship between circulating RBP4 and ApoB-containing lipoproteins in subjects with hypertriglyceridemia. Secondly, the relationships between RBP4 and lipoproteins that were found in the current study cannot confirm the existence of causal mechanisms underlying these associations.

In conclusion, the present study showed that circulating RBP4 was positively correlated with serum levels of ApoB-containing lipoproteins in a steady metabolic state, as well as during a hypocaloric low-fat diet in overweight or obese patients with hypertriglyceridemia. This relationship appears to be attenuated in patients with TG ≥200 mg/dL. Further well designed studies with a greater number of patients are needed to confirm these results and elucidate the underlying mechanisms.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

Clinical and biochemical responses after Gamma Knife surgery for a dopamine-secreting paraganglioma: case report

Constantin Tuleasca,1,2,9 Yves Jaquet,3 Valerie Schweizer,3 Laura Negretti,4 Vera Magaddino,5,9 Philippe Maeder,6,9 Karim-Alexandre Abid,8,9 Benoit Lhermitte,7,9 Eric Grouzmann,*8,9 Marc Levivier*1,9

1Department of Clinical Neurosciences, Neurosurgery Service and Gamma Knife Center, Centre Hospitalier Universitaire Vaudois (CHUV); 2Signal Processing Laboratory (LTS 5), École Polytechnique Fédérale de Lausanne; 3Department of Otolaryngology, Head and Neck Surgery, 4Radiation Oncology Service, 5Institute of Radiation Physics, 6Radiology Department, 7Neuropathology Department, 8Service of Biomedicine, Laboratoire des Catécholamines et Peptides, Centre Hospitalier Universitaire Vaudois (CHUV); 9University of Lausanne, Faculty of Biology and Medicine; Lausanne, Switzerland

*These authors contributed equally to this work

ABSTRACT

INTRODUCTION: The efficacy of Gamma Knife surgery (GKS) in local tumor control of non-secreting paragangliomas (PGLs) has been fully described by previous studies. However, with regard to secreting PGL, only one previous case report exists advocating its efficacy at a biological level. CASE REPORT: The aims of this study were: 1) to evaluate the safety/efficacy of GKS in a dopamine-secreting PGL; 2) to investigate whether the biological concentrations of free methoxytyramine could be used as a marker of treatment efficacy during the follow-up. We describe the case of a 62-year-old man diagnosed with left PGL. He initially underwent complete surgical excision. Thirty months after, he developed recurrent biological and neuroradiological disease; the most sensitive biomarker for monitoring the disease, concentration of plasma free methoxytyramine, started to increase. GKS was performed at a maximal marginal dose of 16 Gy. During the following 30 months, concentration of free methoxytyramine gradually decreased from 0.14 nmol/l (2*URL) before GKS to 0.09 nmol/l, 6 months after GKS and 0.07 nmol/l at the last follow-up after GKS (1.1*URL), confirming the efficacy of the treatment. Additionally, at 30 months there was approximately 36.6% shrinkage from the initial target volume. CONCLUSION: The GKS treatment was safe and effective, this being confirmed clinically, neuroradiologically and biologically. The case illustrates the importance of laboratory tests taking into account methoxytyramine when analyzing biological samples to assess the biochemical activity of a PGL. In addition, the identification of methoxytyramine as a unique positive biomarker could designate it for the monitoring of tumor relapse after treatments, including Gamma Knife surgery.

Key words: Dopamine, Gamma Knife surgery, Paraganglioma, Secreting
INTRODUCTION

Paragangliomas (PGLs) are neuroendocrine tumors, slow growing and arising principally in the head and neck. In 1-2% of cases they comprise functional, secreting catecholamines entities. Considered generally benign, they however include 1-5% malignancies. Due to their topography and local invasion of surrounding structures, they are associated with a complexity of clinical signs and symptoms (headache, pulsatile tinnitus, lower cranial nerve palsies). Therefore, their treatment remains challenging and includes a variety of alternatives, including surgical resection, embolization and radiotherapy. Stereotactic radiosurgery has recently emerged as a non-invasive alternative for the treatment of head and neck PGL, demonstrating high rates of local tumor control and symptoms with minimal associated morbidity.

Functional PGLs secreting catecholamines are rather rare entities. Hypertension is present in 95% of them. While the sensitivities reported in patients with catecholamine-secreting PGLs are 74% for urine total metanephrines, 84% for norepinephrine, 18% for dopamine and 14% for epinephrine, unfortunately no sensitivity has thus far been reported for methoxytyramine. However, it appears that free methoxytyramine is virtually absent in plasma and any presence is associated with an intratumoral metabolism of dopamine into its O-methoxylated form, methoxytyramine by catechol-O-methyltransferase (COMT). In the rare setting of functional PGLs, only one case report of radiosurgical treatment has to date been published.

We present the case of an adult male with a recurrent dopamine-secreting PGL, biochemically monitored by measuring urine and plasma free methoxytyramine, that was treated successfully with GKS for a post-surgical recurrence. The tumor was not producing normetanephrine nor metanephrine.

CASE REPORT

Initial presentation and work-up

A 62-year-old man was referred to an otolaryngologist in July 2004 for investigation of a left cervical mass. After a clinical examination, the otolaryngologist considered the mass to be a lipoma and no biopsy was performed. No other investigation was performed for the next 4 years.

In April 2008, the patient complained of cervical pain and noted an increase of the cervical mass. His general practitioner ordered a computed tomography scan of the neck that revealed a hypervascular 3.5 x 3.8 cm mass in the left carotid region. A fine-needle aspiration biopsy was not conclusive and showed only hemorrhagic material.

In May 2008, a magnetic resonance imaging scan with MR angiography sequences was carried out, which showed a 4 x 4 x 4.7 cm left latero-cervical strongly contrast-enhancing mass posterior to the carotid artery. His medical history was significant for an arterial hypertension diagnosed in June 2002 and treated by an angiotensin II receptor blocker (ARB) and a thiazide diuretic, but no specific symptoms suggestive of catecholamine excess were reported. The patient was scheduled for surgical removal of the mass.

A 123I-Meta-iodobenzylguanidine (123I-MIBG) scintigraphy did not reveal uptake through norepinephrine transporters, a feature previously reported for dopamine-secreting tumors, probably due to the absence of the vesicular monoamine transporter VMAT. 18F-fluoro-D-glucose Positron Emission Tomography (FDG-PET) confirmed the localization of the known mass, which had an increased FDG uptake but did not invade other tissues. PET also revealed a centimetric left jugular lymph node and multiple bone lesions in the thoracic and lumbar spine, all without increased uptake. Findings on a subsequent bone scintigraphy and a total-body MR imaging were not compatible with metastatic lesions.

In August 2008, the patient underwent a gross total resection of the mass. Blood pressure and heart rate were unremarkable throughout the surgical procedure, confirming that the tumor was not secreting vasoactive bioamines and peptides since no changes of blood pressure or heart rate were observed as is the case in the surgical removal of pheochromocytomas. The arterial hypertension still persisted after tumor removal and was adequately controlled by an ARN and a thiazide diuretic, this being indicative of essential hypertension. The histopathological ex-
amination of the tumor confirmed the diagnosis of PGL. The tumor margins reached the resection area on many sites and tumoral cells presented a marked pleomorphism with few mitoses (one mitosis per 10 fields at high magnitude).

The possibility of a biochemically functional tumor was evaluated by 24-hour urine and plasma measurements for metanephrines and catecholamines. A urine specimen collection was also performed.

**Surgical intervention and initial surveillance**

The urine collection showed elevated concentrations of nmol/24h methoxytyramine at 10880 (Upper Reference Limit, URL <1900) and a relatively high concentration of dopamine but still within the normal range at 3222 (URL <3300). Similarly, plasma free and total methoxytyramine and dopamine were increased to 2.78, 26.90 and 20.46 nmol/L (URL <0.06, 2.99 and 0.38, respectively). Norepinephrine and epinephrine and their methoxylated metabolites were within their respective reference intervals in urine and plasma. Routine blood analysis was unremarkable. A new 24h urinary sample confirmed increased levels of methoxytyramine at 12704 and dopamine at 3547 nmol/24h. Genetic testing for mutations of the succinate dehydrogenase genes SDHD and SDHB was negative.

A piece of tumor tissue was used for biochemical analysis and demonstrated an abundant production of dopamine and methoxytyramine (25.3 and 6.4 pmol/mg of wet tissue, respectively), along with low amounts of norepinephrine and epinephrine (0.87 and 0.87 pmol/mg) and of normetanephrine and metanephrine (0.04 and 0.4 pmol/mg), indicative of decreased expression of dopamine beta-hydroxylase, the enzyme that transforms dopamine into norepinephrine.

The patient recovered and 5 days post-surgery biochemical evaluation showed catecholamines and metabolites within normal ranges, as they remained during the last 2.5 years of the patient’s annual follow-up. A left Claude-Bernard-Horner syndrome, with additional partial palsy of the left 11th and 12th nerves, had marked the clinical postsurgical course.

The biological sample analyzed in July 2010 (2 years after surgery) showed a concentration of plasma free methoxytyramine below the URL at 0.05 nmol/l (URL<0.06) and low urine methoxytyramine at 1211 nmol/24 hr (URL at 1900). In February 2011 (2 years and a half after surgery), urinary and plasmatic levels of methoxytyramine were measured and started to increase (1393 and 0.10, respectively). A new FDG-PET was compatible with a recurrence of the PGL in the region of the left carotid and also revealed a lesion in the vocal chords with increased uptake. A MR confirmed a 1.7 x 1 x 1.2 cm lesion, situated in the left retrostyloid parapharyngeal space, which was hyperintense on T2, but no other lesions. Therefore, a neuroradiological and biochemical recurrence was diagnosed.

The anatomical location of the recurrent tumor, and particularly its close proximity to the internal carotid artery, represented a high surgical risk in the event of a new open microsurgical intervention. After multidisciplinary discussion regarding surgical or radiation management, a GKS treatment was decided upon.

**Radiosurgical salvage therapy and postoperative course**

Gamma Knife surgery was performed in December 2011, after placement of the stereotactic Leksell Frame type G, with the Leksell Gamma Knife Perfexion® (Elekta Instruments, AB, Sweden) (Figure 1a). The lesion size was 20 mm (lateral), 19 mm (antero-posterior) and 42 mm (vertical) at the time of GKS. The target volume was 6.64 cc. The maximum marginal dose was 16 Gy at the 50% prescription isodose. The prescription isodose volume was 9.79 cc. The conformity, the selectivity, the Paddick and the gradient index were 1, 1.694, 1.694 and 3.122, respectively. Eighteen isocenters were used (including composite ones). The dose gradient was optimized towards the interface with the ipsilateral internal carotid artery and also towards the jugular foramen, as the tumor was infiltrating the former.

The follow-up course included regular clinical, biochemical and neuroradiological assessment. The latest available information after GKS was at 30 months.

Seven months after GKS, no new symptom was present. There was persistent partial palsy of the left 11th and 12th nerves. Biochemically, the urinary and
plasmatic levels of catecholamines were discretely diminishing. Neuroradiologically, there was stability of the tumor size and aspect.

The patient underwent surveillance imaging, serology and clinical assessment at 19 and 30 months after salvage radiosurgery and remained clinically stable and without new neurologic deficits. Biochemically, the plasmatic concentration of free methoxytyramine gradually declined from 0.14 nmol/l (2*URL) before GKS to 0.07 nmol/l 30 months after GKS (1.1*URL), confirming the efficacy of the treatment (Table 1 and Figure 2). Furthermore, their urine concentration from 1803 (before GKS) was decreased to 1232 nmol/l (at 30 months after GKS), a 31.7% decline compared to pre-surgical values (Figure 2). The MR already showed shrinkage of the tumor at 19 months, which persisted over time (Figure 1b). At the last MRI, the remnant tumor volume measured 4.21 cc, which corresponded to a shrinkage of approximately 36.6% from the initial target volume (as calculated in the Leksell GammaPlan station, Elekta Instruments, AB, Sweden, the same as for the measurement of the initial GKS target volume).

**Table 1.** Measured plasma free and urine methoxytyramine concentrations during monitoring of the disease, urinary levels, nmol/24h

<table>
<thead>
<tr>
<th>Date</th>
<th>Plasma free methoxytyramine</th>
<th>Urine methoxytyramine</th>
</tr>
</thead>
<tbody>
<tr>
<td>July 2008 (Before surgery)</td>
<td>2.78</td>
<td>12704</td>
</tr>
<tr>
<td>September 2008 (Immediate post surgery)</td>
<td>&lt;0.03</td>
<td>617</td>
</tr>
<tr>
<td>July 2010 (16 months before GKS)</td>
<td>0.05</td>
<td>1211</td>
</tr>
<tr>
<td>February 2011 (10 months before GKS)</td>
<td>0.10</td>
<td>1393</td>
</tr>
<tr>
<td>March 2011 (9 months before GKS)</td>
<td>0.14</td>
<td>1803</td>
</tr>
<tr>
<td>June 2012 (6 months post-GKS)</td>
<td>0.09</td>
<td>1271</td>
</tr>
<tr>
<td>June 2013 (18 months post-GKS)</td>
<td>0.06</td>
<td>1329</td>
</tr>
<tr>
<td>June 2014 (30 months post-GKS)</td>
<td>0.07</td>
<td>1232</td>
</tr>
</tbody>
</table>
In this context, the need for minimally invasive therapies appeared. Embolization is not considered curative due to formation of anastomotic blood vessels and is recommended only as a preparation for surgery.16 Meanwhile, external-beam radiotherapy presents the risk of serious complications due to large fields size, as suggested by Li et al.17

Gamma Knife surgery emerged as an alternative during the last decade due to its minimal invasiveness and local tumor control rates comparable to surgical strategies, while preserving or even improving cranial nerve function.4-6,18

Recently it has been reported that functioning PGLs also respond to stereotactic radiosurgery, and in particular to GKS, at both the biochemical and neuroradiological level.3 In addition, previously published papers have suggested that conventional doses and techniques of radiotherapy are not sufficient to adequately control these particular and rare cases.19-21

Functioning PGLs are very rare in the head and neck, although they are known to display neuroendocrine activity through secretion of catecholamines.10,22 The sensitivities reported in patients with catecholamine-secreting PGLs are 74% for urine total metanephrines, 84% for norepinephrine, 18% for dopamine and 14% for epinephrine, but unfortunately no sensitivity has as yet been reported for methoxytyramine.8 A recent study on a cohort of 136 patients with PGLs showed catecholamines secretion only in 23%.23 The isolated secretion of methoxytyramine by PGLs is limited to

**DISCUSSION**

Head and neck PGLs arise mostly sporadically, but they can also be familial (10-15% of cases).10 They can be found in 3 main areas: most commonly around the bifurcation of the common carotid artery, less commonly within or along the inferior nodose ganglion of the 5th cranial nerve and, lastly, also along the branches of 4th and 5th nerve, in close association with the temporal bone, primarily in and around the jugular foramen (these latter known as glomus jugular tumors or chemodectoma). Glomus jugular tumors are extremely rare, with an incidence of about 1 in 30,000 in the healthy population, representing 0.0012% of all tumors diagnosed in humans.7 They are usually slow growing, with a mean growth rate of 0.8 to 1 mm per year.11 Glomus jugular tumors are highly vascular tumors; they grow expansively, eroding the jugular foramen and the petrous bone and they commonly gain access to the subarachnoid space by penetrating the dura of the posterior fossa or, less commonly, the middle fossa.12

The first surgical excision for this type of tumor was performed in 1945 by Rosenwasser.13 During the last few decades, progress in the surgical treatment of these tumors has been marked by some controversy due to significant morbidity associated with complete surgical excision,12,14 including up to 59% postoperative cranial nerve deficits.12 In subtotal resections, as reported by several studies, up to 15% recurrences are reported.12,14,15

In this context, the need for minimally invasive therapies appeared. Embolization is not considered curative due to formation of anastomotic blood vessels and is recommended only as a preparation for surgery.16 Meanwhile, external-beam radiotherapy presents the risk of serious complications due to large fields size, as suggested by Li et al.17

Gamma Knife surgery emerged as an alternative during the last decade due to its minimal invasiveness and local tumor control rates comparable to surgical strategies, while preserving or even improving cranial nerve function.4-6,18

Recently it has been reported that functioning PGLs also respond to stereotactic radiosurgery, and in particular to GKS, at both the biochemical and neuroradiological level.3 In addition, previously published papers have suggested that conventional doses and techniques of radiotherapy are not sufficient to adequately control these particular and rare cases.19-21

Functioning PGLs are very rare in the head and neck, although they are known to display neuroendocrine activity through secretion of catecholamines.10,22 The sensitivities reported in patients with catecholamine-secreting PGLs are 74% for urine total metanephrines, 84% for norepinephrine, 18% for dopamine and 14% for epinephrine, but unfortunately no sensitivity has as yet been reported for methoxytyramine.8 A recent study on a cohort of 136 patients with PGLs showed catecholamines secretion only in 23%.23 The isolated secretion of methoxytyramine by PGLs is limited to

**Figure 2.** Plasma concentration (left panel) of free methoxytyramine gradually declined from 0.14 nmol/l (2*URL) before GKS to 0.07 nmol/l and 30 months after GKS (1.1*URL); its urine concentration (right panel) decreased from 1803 (before GKS) to 1232 nmol/l (at 30 months after GKS), a 31.7% change compared with the values before GKS.
8-13 % of these tumors.\textsuperscript{23,24} Thus, the tumors under discussion represent a real challenge for biochemical diagnosis, since medical laboratories usually do not report dopamine and its methoxylated derivative in plasma and urine samples. Therefore, chromatograms must be carefully inspected in order not to miss the diagnosis. In the present case, urinary dopamine appeared initially, at diagnosis, to be negative in one sample and slightly above the URL in a second sample, contrasting with the clear-cut increases above URL of plasma free (46-fold) and total methoxytyramine (9-fold), urine methoxytyramine (6-fold) and plasma dopamine (54-fold), in agreement with previous observations.\textsuperscript{23,24} Urinary dopamine deriving from renal extraction and decarboxylation of circulating 3,4-dihydroxyphenylalanine, found in certain kinds of foods and herbs, and by L-DOPA treatment is common in patients treated for Parkinson’s disease, thus leading to false positive results.\textsuperscript{25} The higher diagnostic sensitivity of methoxytyramine for HNPGL is due to the fact that this tumor expresses catechol-O-methyl-transferase, an enzyme that O-methylates intra-tumoral dopamine into methoxytyramine.

CONCLUSION

The present case illustrates the important need for laboratory tests to take into account methoxytyramine when analyzing biological samples to assess the biochemical activity of a PGL. In addition, the identification of methoxytyramine as being the only positive biomarker in such patients could designate it as a valuable biomarker for the monitoring of tumor relapse.

The GKS treatment was safe and effective, which was confirmed clinically, neuroradiologically and biochemically. To the best of our knowledge, this is the first report using methoxytyramine for the biological follow-up course of the treatment.

SUPPORT

Lausanne University Hospital.

CONFLICT OF INTEREST

The authors report no conflict of interest.

REFERENCES

Multiple endocrine neoplasia type 1 associated with a new germline Men1 mutation in a family with atypical tumor phenotype

Nikolaos Perakakis,1 Felix Flohr,2 Gian Kayser,3 Oliver Thomusch,4 Lydia Parsons,1,5 Franck Billmann,4 Ernst von Dobschuetz,6 Susanne Rondot,7 Jochen Seufert,1 Katharina Laubner1

1Division of Endocrinology and Diabetology, Department of Internal Medicine II, University Hospital of Freiburg, Freiburg, Germany; 2Department of Internal Medicine I, St. Vincentius-Hospital, Karlsruhe, Germany; 3Department of Pathology, Institute of Surgical Pathology, University Hospital of Freiburg, Freiburg, Germany; 4Division of Endocrine Surgery, Department of General and Visceral Surgery, University Hospital of Freiburg, Germany; 5School of Biosciences, Cardiff University, Cardiff, United Kingdom; 6Division of Endocrine Surgery Hospital Reinbek St. Adolf-Stift Reinbek, Germany; 7Endocrine Practice and Molecular Laboratory, Heidelberg, Germany

ABSTRACT

BACKGROUND: Multiple endocrine neoplasia type 1 (MEN1) is an autosomal-dominant hereditary disorder associated with the development of endocrine tumors due to reduced expression of the tumor suppressor protein menin. Recent studies indicate a general role of menin in carcinogenesis, affecting the prevalence and clinical course of common non-endocrine tumors such as breast cancer, hepatocellular carcinoma and melanoma. Here we report a new germline missense mutation of Men1 in a German family with atypical tumor phenotype over three generations. Based on the type of mutation, we discuss possible changes in menin function leading to atypical tumorigenesis and present the clinical significance of such findings.

PRESENTATION: A German family with a history of primary hyperparathyroidism presented to our Hospital for further evaluation. Members of the family demonstrated many different atypical tumors, such as renal cell carcinoma, papillary thyroid cancer and prostate cancer. DNA sequencing from peripheral blood revealed a novel mutation: Ser38Cys [TCC>TGC] in exon 2, codon 38 of Men1. This novel mutation is located in a region of menin which is responsible for interactions with the transcription factor JunD. This factor has recently been associated with prostate cancer. DNA sequencing of two of the atypical tumors (prostate cancer, papillary thyroid cancer) did not reveal a loss of heterozygosity, indicating an impact on menin expression and function in the heterozygous state, in line with results in +/-Men1 mutant mice developing prostate cancer.

CONCLUSION: The results and clinical course of disease in this case indicate the potential role of menin in the development of non-endocrine or atypical-endocrine tumors in MEN1 patients. Further investigations are needed to clarify both the general role of menin and the importance of specific mutations in carcinogenesis. Nevertheless, in families with uncommon manifestations of the syndrome early diagnostic adjustments should be considered.

Key words: Atypical tumors, MEN1, New mutation, Prostate cancer

Address for correspondence:
Dr. Nikolaos Perakakis, MD, Division of Endocrinology and Diabetology, Department of Internal Medicine II, University Hospital of Freiburg, 55 Hugstetter Str., 79106 Freiburg, Germany; Tel.: +4976127034200, Fax: +4976127034130, E-mail: nikolaos.perakakis@uniklinik-freiburg.de
Received: 28-05-2015, Accepted: 09-09-2015
INTRODUCTION

Multiple endocrine neoplasia type 1 (MEN1) is a rare autosomal-dominant hereditary endocrine tumor syndrome associated with reduced levels of the tumor suppressor protein menin. Results from clinical studies and preclinical experiments indicate an additional involvement of *Men1*, which encodes menin, in the development of non-endocrine tumors. Herein we report a novel mutation of *Men1* followed over three generations of a German family with atypical non-endocrine tumor entities.

CASE DESCRIPTION

A 75-year-old man (patient 1) presented to our hospital with his 47-year-old son (patient 2) with primary hyperparathyroidism (PHPT). Patient 1 had been previously diagnosed with: a) papillary thyroid cancer with multiple thyroid nodules at the age of 72, treated with total thyroidectomy and radiation, b) renal cell carcinoma at the age of 72, treated with partial kidney resection, c) prostate cancer at the age of 62, treated with prostatectomy and d) PHPT at the age of 72, treated with total parathyroidectomy. At the first visit we detected elevated chromogranin A levels. Gastroscopy, colonoscopy, repeated DOTATATE-PET-CTs and annual abdominal MRIs did not locate a gastrointestinal tumor. An MRI of the pituitary gland at the fifth annual follow-up revealed a microadenoma, classified as a non-secreting tumor by inconspicuous hormonal results. Additionally to PHPT diagnosed at the age of 44, patient 2 was diagnosed with: a) cold thyroid nodule at the age of 35, treated with total thyroidectomy, and b) ulcerative colitis at the age of 42. PHPT was treated initially with partial parathyroidectomy and on relapse with total parathyroidectomy and autotransplantation of one parathyroid gland to the brachioradialis muscle. Furthering the family history, the brother of patient 2 (patient 3) and daughter of patient 3 (patient 4) were both diagnosed at the first screening for MEN1 syndrome with PHPT at the ages of 46 and 21, respectively, and subsequently operated on. Interestingly, patient 3 was also diagnosed with prostate cancer at the age of 46 and was treated with prostatectomy (Figure 1). Patients 3 and 4 had follow-up visits in another clinic.

The genetic diagnosis of peripheral blood from patients 1 and 2 revealed a novel heterozygous missense mutation: Ser38Cys (TCC>TGC) in exon 2, codon 38 of *Men1* (Figure 2). Additional analysis by DNA sequencing and multiplex ligation-dependent probe

![Figure 1](image1.png)  
**Figure 1.** Patients 1-4 developed a PHPT and patients 1 and 3 cold thyroid nodules. Patients 1 and 3 additionally developed prostate cancer early in life. Patient 1 was also diagnosed with a papillary thyroid cancer and a renal cell carcinoma.

![Figure 2](image2.png)  
**Figure 2.** A novel missense mutation in *Men1/Exon 2* c.113>G, p.Ser38Cys associated with endocrine and very probably atypical non-endocrine tumor entities over three generations of a German family.
amplification from the papillary thyroid carcinoma of patient 1 and from the prostate cancer of patient 3 did not demonstrate a “Loss of Heterozygosity” (LOH).

**DISCUSSION**

The management of families with MEN1 syndrome is often very demanding. The clinical guidelines concentrate on the early detection of the three most common tumor manifestations, namely parathyroid adenoma, enteropancreatic neuroendocrine tumors and anterior pituitary adenomas. Other features are either rare (e.g. adrenal cortical tumors) or clinically less important (e.g. lipomas, angiofibromas). However, different atypical tumor entities have occasionally been described in families with MEN1 syndrome, raising the following questions. Does menin have a major role in carcinogenesis, including a non-endocrine function? Do some MEN1-families with specific mutations have an increased risk of non-endocrine tissue malignancies, which may demand diagnostic adjustments?

In vivo, Men1 mice die embryonically, while Men1 mice develop mostly endocrine tumor disorders, such as parathyroid hyperplasia, and pancreatic, pituitary and adrenal cortical tumor. Menin is however expressed not only in endocrine tumors but also in many non-endocrine human cancer cells, such as in breast, cervical, brain glioma and gastric cancer cell lines. Furthermore, changes in menin expression have recently been related to the development of non-endocrine tumors. A downregulation of menin is observed in osteosarcoma tissues. Additionally, MEN1 patients more frequently develop malignant melanomas. Menin suppresses the expression of the growth factors pleiotrophin and phosphatidylinositol 3-kinase, inhibiting melanoma cell proliferation and migration. Moreover, menin stimulates the transcription of genes involved in DNA repair. By contrast, increased menin levels promote hepatocellular carcinogenesis correlating negatively with the overall and tumor-free survival rates. In our case, two of the three male carriers of the Men1 mutation developed prostate cancer early. Sequencing of prostate tumor cells from patient 3 excluded LOH, which could explain these early manifestations. However, a heterozygous status may also contribute to carcinogenesis. Men1 mutant mice develop prostate cancer due to drastically reduced expression of menin in the carcinogenic tissue. Despite this, only some tumor tissues demonstrate LOH. Furthermore, a study using DNA sequencing of formalin-fixed human prostate cancer biopsies revealed a missense mutation of Men1. Finally, neuroendocrine tumors from MEN1 patients express genes, such as FGF9, which can stimulate the growth of pre-existing prostate cancer. Similar observations have been reported in women with breast cancer, although the role of menin in these cases seems more complicated. In sporadic breast cancer, Men1 mRNA is overexpressed and probably stimulates tumor proliferation by regulation of estrogen receptor alpha activity. Interestingly, a Men1 somatic mutation is rarely detected in these tumors. In contrast, females with MEN1 syndrome, who have reduced Men1 mRNA expression, demonstrate an unexpectedly increased risk of invasive breast cancer. This can be partially explained by the higher frequency of LOH in these cases compared to sporadic breast cancer. However, just one third of these tumors demonstrate LOH, although in most of them the nuclear localisation of menin is severely reduced. This indicates a more complex mechanistic pathway for menin, which has not been completely described as yet. To conclude, it seems that menin has a general role in carcinogenesis, including functions in non-endocrine organs that may previously have been underestimated.

A second important question is if the tumor manifestation or the course of the disease depends on the type of mutation. Previous studies have not shown a genotype-phenotype association. However, novel studies dispute this point. Firstly, the type of Men1 mutation can lead either to a clinically overt MEN1 syndrome or to milder clinical conditions. For example, familial isolated hyperparathyroidism (FIHP) is characterised by the development of PHPT alone and is associated with specific Men1 mutations, often missense (in approximately 40% of the cases) and only occasionally nonsense or frameshift (30% of the cases) mutations. Conversely, in MEN1 syndrome with a classical phenotype, a nonsense or frameshift mutation, which leads to a truncated and consequently inactivate protein, is present in 65% of cases. Although the reported mutation here is a missense one, the family meets the criteria for MEN1 syndrome according to
the international guidelines. Subject 1 has developed two typical MEN1 manifestations (PHPT, pituitary adenoma), while subjects 2 and 3 (first degree relatives of subject 1) have developed PHPT. Finally, subject 4 carries the same mutation and was diagnosed with PHPT at the first screening for MEN1 at the age of 21, relatively early for FIHP. As subject 1 was the first to be diagnosed with MEN1, the late diagnosis of PHPT at the age of 72 in this subject can be explained by the lack of early screenings. The type of mutation can often affect the course of the disease. For example, patients with Men1-mutations resulting in loss of interaction with the checkpoint kinase 1-interacting domain have a higher risk of malignant pancreatic neuroendocrine neoplasia with aggressive course of disease and higher rates of disease-related death. Moreover, patients with mutations affecting the JunD-interacting domain have an increased risk of death secondary to a typical MEN1 tumor, demanding a more aggressive therapeutic approach. The novel Men1 mutation reported here is located in codon 38 of exon 2. Exon 1 is not translated and most of the mutations in exon 2 are frameshift or nonsense mutations leading to truncated menin. However, the current mutation leads to a change in one amino acid (from serine to cysteine). The first 40 amino-acids at the N-terminal end of menin are responsible for interactions with the transcription factor JunD. This novel mutation may therefore affect the function of the Menin-JunD complex. Interestingly, JunD has been linked to progression of prostate cancer due to overproduction of reactive oxygen species in cancer cells. Taking into consideration the family history and possible associations between MEN1 and prostate carcinogenesis, we recommended urological evaluation and annual biochemical screening (PSA) to patient 2.

CONCLUSION

We describe a new germline mutation of Men1 in a German family, which is associated with an atypical phenotype. Since the role of menin as a tumor regulator in many organs remains to be established, it is difficult to distinguish between random observations and etiological relations in MEN1 families with atypical tumors. For this reason, situational adaptations of the recommended diagnostic procedure and follow-up should be considered. Future studies should focus on the potential effect of Men1 mutations on the development and progression of common but in MEN1 patients rarely described non-endocrine tumors.

DISCLOSURE STATEMENT

The authors have nothing to disclose.

REFERENCES

Intractable hypoglycaemia in a patient with advanced carcinoid syndrome successfully treated with hepatic embolization

Angelos Kyriacou,1 Was Mansoor,2 Jeremy Lawrance,3 Peter J. Trainer1

1Department of Endocrinology; 2Department of Medical Oncology; 3Department of Radiology, The Christie NHS Foundation Trust, Manchester Academic Health Science Centre; Manchester; UK

ABSTRACT
A male patient presented at the age of 54 years with metastatic pancreatic neuroendocrine tumour (NET). He was managed with interferon and multiple courses of MIBG therapy which controlled his disease for about seven years. He then developed symptomatic hypoglycaemia which resolved with the introduction of somatostatin analogue treatment and further therapeutic MIBG. However, three years later he was admitted to hospital with severe and intractable hypoglycaemia, which persisted despite treatment with dietary manipulation, diazoxide, long-acting octreotide injections, intravenous infusion of dextrose and octreotide and everolimus. Bland hepatic embolization was attempted as a last resort and resulted in prompt and dramatic improvement of his condition with no hypoglycaemia for five months. We recommend that hepatic embolization should be considered in patients with advanced and metastatic NETs accompanied by refractory hypoglycaemia, with the aim of symptomatic relief and palliation, and possibly some survival benefit.

Key words: Hepatic embolization, Hypoglycaemia, Neuroendocrine tumour

INTRODUCTION
Pancreatic neuroendocrine tumours (pNETs) are rare, with an annual incidence estimated at 2–4 per 1,000,000 population, although given their indolent nature this figure could be an underestimate.1 Surgery is considered as first line treatment for pNETs, but it is often not possible at presentation due to extensive and metastatic disease; for patients who cannot have surgery, the therapeutic options include somatostatin analogues (SA), targeted therapies (e.g. sunitinib and everolimus), chemotherapy and liver-directed therapies.1 pNETs are divided into functional and non-functional (tumours that are hormonally-secreting or not, respectively).

Hypoglycaemia in the context of metastatic pNETs is due to either functional transformation of the tumour2 or, alternatively, to depletion of hepatic glycogen stores with extensive hepatic metastases. The usual treatment algorithm for hypoglycaemia in such instances includes the use of dietary manipulation, diazoxide and SA. Dietary manipulation involves the intake of small, but multiple, meals with a low glycaemic index. Diazoxide inhibits insulin release; however, its use is limited by frequent adverse effects, especially fluid retention and hirsutism. Octreotide, a synthetic SA, has affinity for sst2 and sst5 receptors and can improve
Intractable hypoglycaemia treated with hepatic embolization

fasting blood glucose and insulin in both benign and malignant insulinomas for up to three years. Moreover, the PROMID Study Group has demonstrated in a double-blinded, prospective, randomized-controlled trial that octreotide LAR significantly delays time to tumour progression in patients with metastatic midgut NETs, regardless of functionality-status. The most favourable outcomes were observed in those patients with low hepatic tumour load and with resection of the primary tumour.

Despite the above “conventional” measures in nonoperable NETs, some patients do not respond, some cannot tolerate the aforementioned therapies due to side effects and others experience relapsing hypoglycaemia, after a period of symptom control. In such cases options include radiolabelled somatostatin analogues, such as Lutetium-177 (with/without capecitabine), mTOR receptor inhibitors, such as everolimus, or chemotherapy, such as streptozotocin-5FU or capecitabine-temozolamide.

We therefore present our experience in treating a patient with refractory hypoglycaemia due to a metastatic pancreatic carcinoid neuroendocrine tumour (NET); his condition became unresponsive to any of the ‘conventional’ hyperglycaemic therapies and, indeed, everolimus; instead, he showed a remarkable response to hepatic embolization.

SUBJECTS AND METHODS

Clinical case

A 54-year old man presented in 2002 with abdominal pain, but no symptoms suggestive of carcinoid syndrome or hypoglycaemia. A computed tomography (CT) scan showed a pancreatic mass with retroperitoneal and mediastinal lymph nodes and the presence of multiple liver and bone metastases. His urinary 5 hydroxy-indoleacetic acid (5HIAA) level was raised. A CT guided biopsy of the retroperitoneal mass revealed a well-differentiated NET of the pancreas (MIB-1 score <2%).

Given the burden and spread of disease, surgery was deemed inappropriate. Instead, the patient was commenced on interferon-2α therapy. A meta-iodo-benzylguanidine (MIBG) scan done in 2004 showed increased uptake in the lower abdomen as well as in the known metastatic deposits. Interferon-2α therapy was discontinued and between March 2005 and October 2009 he received four fractions of iodine-131 MIBG. Abdominal and bone pain plus occasional flushing were well controlled, albeit requiring continued supportive therapy with opioids and zolendronic acid. Radiologically, there was a trend towards progression of liver disease.

In September 2009, he developed symptomatic hypoglycaemia, associated with episodes of loss of consciousness, which was confirmed biochemically. In March 2010, he commenced octreotide LAR 20 mg every four weeks and received a fifth fraction of Iodine-131 MIBG therapy, which resulted in resolution of his hypoglycaemic episodes. Octreotide LAR controlled his hypoglycaemic symptoms until February 2013. At this point, hypoglycaemic symptoms were paralleled by tumour progression and a general deterioration in his condition. Diazoxide (150 mg daily) was added to octreotide but he continued to have increasingly frequent hypoglycaemic episodes culminating in a severe, intractable hypoglycaemic attack, necessitating hospital admission. Despite continuous intravenous infusions (IVI) of 10% dextrose and octreotide (300 µg/24hrs) he had persistent hypoglycaemia. The addition of everolimus (10 mg once daily) was of no benefit.

In view of the large volume of hepatic disease (Figure 1), bland left hepatic artery embolization (Figure 2) with 1.5 mls of Spherical Polyvinyl Alcohol (Embozene® Color-Advanced Microspheres, CeloNova BioSciences, Inc., San Antonio, USA), containing 400 microns, was given and resulted in a dramatic reduction in the frequency and severity of hypoglycaemia within 24 hours.

His octreotide and glucose IVI were discontinued within 72 hours. The patient was discharged home on octreotide LAR 30 mg and everolimus 10 mg; the latter was continued for its potential anti-tumour action. He remained asymptomatic with no hypoglycaemic episodes for five months before hypoglycaemia recurred and he died two weeks later.

DISCUSSION

Our case demonstrates that with disease progression in metastatic pNETs it is often not possible to
The cause of the recurrent hypoglycaemia in our patient is likely to relate to the development of inappropriate insulin co-secretion from his NET (as previously described) and/or depletion of his hepatic glycogen stores as the disease evolved, but, unfortunately, insulin and C-peptide were not measured. Hypoglycaemia due to glucagon-like peptide 1 (GLP-1) secretion has also been reported in a patient with a strumal ovarian carcinoid syndrome; co-secretion of somatostatin by the same tumour meant that the patient was also suffering with (predominantly fasting) hyperglycaemia.

As Besser et al have reported, the hyperglycaemic “adverse-effect” of everolimus can be utilized in the management of hypoglycaemia secondary to NET. In addition, everolimus has been shown in a randomized-controlled trial to significantly lengthen progression-free survival among patients with progressive advanced pNETs whilst being well tolerated. Our patient was not fit for surgery and, given the lack of benefit from everolimus, his frailty (performance score of 3) and personal preference, chemotherapy was not appropriate.

Radiolabelled peptides (RP) can be used to treat recurrent disease refractory to other treatment modalities. Van Schaik et al have described resolution of hypoglycaemia with 177Lu octreotate and 111In octreotide in patients with metastatic insulinomas. This treatment modality was not offered, in view of the fact that his lesions did not display any uptake on somatostatin receptor scintigraphy.

Hepatic artery embolization (HAE) can take several forms. We used PVA into the left artery as a means of inducing intra-tumoural ischaemia, alternative forms being chemoembolization (HACE) and selective internal radiotherapy (SIRT). Bland embolization improves symptoms, tumour markers and radiological appearances. HACE delivers chemotherapy (e.g. with doxorubicin) attached to the embolic agent. It achieves about a twenty-fold higher chemotherapy concentration comparative to systemic chemotherapy with reduced risk of systemic adverse events. However, there is a dearth of evidence that HACE has a survival benefit compared to bland embolization. Both therapies often require multiple treatment courses; indeed, we were hoping to re-treat our patient if his overall general state improved, but this never materialized.

**Figure 1.** Coronal CT scan of the abdomen performed pre-embolization showing extensive hepatic metastases, some of which are necrotic. A left paraspinal mass is also present on this image. The left hepatic lobe was embolized in preference, given that it contained more tumour bulk and the metastatic lesions were solid rather than cystic and, therefore, more likely hormonally active.

**Figure 2.** Hepatic artery embolization. Left hepatic arterial angiogram captured during embolization of the left hepatic artery, showing the left, right hepatic and gastroduodenal arteries. Note that the catheter needs to be advanced beyond the gastroduodenal artery to avoid embolization of the pancreas, stomach and duodenum.

*signifies the site of injection of the microspheres (a microcatheter is inserted via a guiding catheter).

achieve symptomatic control with ‘traditional’ treatment modalities. Newer therapies should be considered, which in the case of symptomatic and refractory hypoglycaemia include everolimus, radiolabelled peptides and hepatic embolization.
Normal hepatic tissue is very radiosensitive, which precludes conventional external beam radiotherapy, but SIRT combines embolization with delivery of tumour localized radiation in the form of microspheres of glass or resin labelled with $^{90}$Y (a pure beta emitter) to deliver radiation directly into the hepatic artery, via a percutaneous transcatheter technique.\textsuperscript{10,11} It may offer a biochemical and radiological advantage over other forms of embolization and possibly increases survival, though it requires more focused delivery and is expensive.\textsuperscript{10} SIRT was not offered to our patient because by the time this technology was available, our patient was very frail, but also for reasons of patient preference and lack of proven efficacy in the context of NETs.

More recently, a Japanese team have also documented the utility of hepatic embolization in a 45-year old woman with newly diagnosed and aggressive (Ki67 labelling index of 27\%) metastatic malignant insulinoma with hypoglycaemia refractory to dietary manipulation, dextrose IVI and diazoxide.\textsuperscript{12} Unlike our case, she was not given everolimus nor any chemotherapy (at any stage pre-embolization) and, although octreotide was attempted, it was promptly discontinued because of an allergic reaction.\textsuperscript{12} Other notable differences from our case were the two courses of embolization (one on either hepatic lobe) that were applied ten days apart;\textsuperscript{12} we performed half, rather than whole, liver embolization as the latter carries a significant risk of liver failure. Similarly to our case, there was rapid response to hepatic embolization (within 24 hours), albeit more short-lived (hypoglycaemia recurred after 1.5 months).\textsuperscript{12}

This case illustrates the value of hepatic embolization in relieving hypoglycaemia, therefore allowing this patient to live his last five months at home, free from any hypoglycaemic attacks. Hepatic embolization is a cost-effective treatment that should be considered for symptomatic relief in patients with inoperable hepatic metastases causing refractory hypoglycaemia or other hormone-related symptoms.

DECLARATION OF INTEREST

All authors declare that they do not have any conflict of interest.

FUNDING

No funding was required for this study.

CONSENT

Written consent was obtained from the patient’s next of kin.

REFERENCES

TSH-secreting pituitary adenomas treated by gamma knife radiosurgery: our case experience and a review of the literature

Zadalla Mouslech,1 Maria Somali,2 Anastasia Konstantina Sakali,2 Christos Savopoulos,1 George Mastorakos,2 Apostolos I. Hatzitolios1

11st Medical Propedeutic Dept of Internal Medicine, AHEPA University Hospital, Aristotle University of Thessaloniki, Thessaloniki, Greece, 2Department of Endocrinology, Metabolism and Diabetes, Aretaieio Hospital, School of Medicine, University of Athens, Athens, Greece

ABSTRACT

A 43-year-old woman, previously misdiagnosed as having primary hyperthyroidism and treated with antithyroid drugs, presented to us with overt hyperthyroidism, high levels of thyroid hormones and elevated thyroid-stimulating hormone (TSH). Magnetic resonance imaging (MRI) revealed a pituitary microadenoma extending suprasellarly. The patient responded favorably to initial treatment with somatostatin analogs for 2 years but due to the escape phenomenon, TSH levels escalated and hyperthyroidism relapsed. Transsphenoidal adenomectomy was applied but recurrence was again observed due to incomplete tumor removal. Gamma knife radiosurgery was finally employed 5.5 years ago, resulting in complete disease remission without evidence of long-term complications to date. Thyrotropin-secreting adenomas (TSHomas) are rare with an estimated prevalence of about one case per million. We retrieved from the literature 14 cases of TSHomas treated by gamma knife radiosurgery and compared the outcomes. Our results demonstrate the efficacy and safety of gamma knife radiosurgery for achieving remission in most of the cases, suggesting validation of this technique as an effective treatment option for the management of recurrent TSHomas.

Key words: Thyrotropin-secreting pituitary adenoma, Hyperthyroidism, Long-acting somatostatin analogs, Transsphenoidal adenomectomy, Gamma knife radiosurgery, Long-term remission

INTRODUCTION

Among pituitary adenomas, thyrotropin-secreting adenomas (TSHomas) have the lowest prevalence, accounting for 0.5-2.8% of all pituitary adenomas.1-4 The first case was reported in 1960 by Jailer and Holub5 and since then just over 450 cases have been published in the literature. About 70% of TSHomas are pure adenomas, while the rest are mixed, co-secreting mainly growth hormone (GH) and prolactin (PRL).6 The treatment of choice for TSHomas is surgical...
removal of the tumor by the transsphenoidal or subfrontal approach. However, radical removal is usually impossible due to invasion of surrounding structures and to the fibrous nature of these tumors, resulting in high recurrence or failure rates of 47-78%.7-10 If surgery is contraindicated or if it fails to cure, the European Thyroid Association guidelines strongly recommend treatment with somatostatin analogs (SSA), such as octreotide and lanreotide, which have proved to be highly effective in a series of studies.9,18 However, there are cases where manifestation of adverse effects, resistance to or escape from the inhibitory effect of SSAs lead to failure of treatment.15,18,19 In these cases stereotactic irradiation can be employed.11

Stereotactic irradiation can be delivered either in multiple sessions (fractionated stereotactic radiotherapy or FSRT) or in a single session (stereotactic radiosurgery or SRS). Today, gamma knife (GK) is the most widespread radiosurgical method. Its accuracy is comparable to a surgical blade, though it should not be employed for targets bigger than 4 cm since they need larger irradiation beams, producing more damage to surrounding structures. Although GK has to date been used successfully in the treatment of pituitary tumors for almost 50 years (a review by Koga et al20 reports the sizeable number of 38,553 pituitary adenomas treated by gamma knife up to 2008), to our best knowledge only 14 cases of TSHomas treated by GK have been reported in the literature.7,9,21-25 Due to the small number of reported cases and their scant follow-up, few data are available on the long-term efficacy and safety of this technique in the treatment of TSHomas. Here, we describe a case of a recurrent TSHoma where gamma knife radiosurgery induced long-term control of the disease and we review the literature on the outcome of TSHoma cases treated by the same means.

CASE REPORT

A 43-year-old woman presented in 2002 to our department complaining of palpitations. Eight years earlier she had been diagnosed with primary hyperthyroidism and treated with antithyroid drugs ever since. She reported recurrence of hyperthyroidism following every attempt to discontinue medical treatment.

On physical examination she had tremor, extra-systolic arrhythmia and a palpable thyroid gland. Hormonal assessment revealed high free circulating thyroid hormones (FT₄=32pmol/L, n.v. 11-25 and FT₃=12pmol/L, n.v. 3.3-8.2) and high TSH concentrations (TSH=12.5mIU/L, n.v. 0.3-4). The rest of the anterior pituitary hormones were within normal range. The response of TSH to TRH stimulation test was blunted (TSH 0min=11.5, TSH 30min=15.3, and TSH 60min=13.5mIU/L), suggesting the presence of a TSHoma. Thyroid ultrasonography demonstrated diffuse enlargement of the thyroid gland and magnetic resonance imaging (MRI) confirmed the presence of a pituitary microadenoma of 9 mm in diameter which extended suprasellarly (Figure 1). A pituitary scintigraphy with radiolabeled octreotide revealed high uptake of the octreotide by the pituitary gland, thus confirming the presence of somatostatin receptors.

The patient refused surgical removal of the tumor and was initially treated with a combination of lanreotide and antithyroid drugs. Lanreotide was started at a dose of 60 mg once monthly which was gradually escalated to 120 mg. Consequently, a euthyroid state was restored and the tumor shrank by 2 mm. These favorable results lasted up to 2 years, at which point, in 2004, the hyperthyroidism relapsed indicating that TSH secretion had escaped from the inhibitory effect of the somatostatin analog. Tumor size steadily increased and by 2006 it again measured 9 mm in diameter in MRI visualization, while TSH, FT₄ and FT₃ levels averaged 30.39mIU/L, 34pmol/L and 13pmol/L, respectively (Figure 2). After a sudden episode of paroxysmal atrial fibrillation, which resulted in hospitalization, the patient gave consent and underwent endonasal transsphenoidal adenomectomy on 29/6/2006. Postoperative MRI revealed a residual tumor mass of 3 mm in diameter, while TSH, FT₄ and FT₃ levels averaged 30.39mIU/L, 34pmol/L and 13pmol/L, respectively (Figure 2). After a sudden episode of paroxysmal atrial fibrillation, which resulted in hospitalization, the patient gave consent and underwent endonasal transsphenoidal adenomectomy on 29/6/2006. Postoperative MRI revealed a residual tumor mass of 3 mm in diameter, while postoperative TSH decreased to 5mIU/L. Histological examination by immunohistochemistry proved positive for TSH and negative for GH, PRL, FSH, LH and ACTH, confirming the diagnosis of a pure TSHoma. Further molecular and pathological analysis, such as the determination of the Ki-67 signaling index, was not conducted.

One year later TSH concentrations had increased to 9.0mIU/L with FT₃ and FT₄ values within the upper normal range. Alternative treatment with radiosurgery was proposed to the patient, which was initially
refused. While waiting for her decision whether or not to undergo radiosurgery the patient became hyperthyroid. We chose to administer antithyroid drugs based on our previous experience with lanreotide, which proved to be inefficient in this patient in the long run. In 2009, TSH reached its highest ever level of 78mIU/L, while in the MRI visualization the size of the residual adenomatous tissue had slightly increased to 0.4x0.2 cm. In May 2010, the patient finally underwent stereotactic radiosurgery with Leksell Gamma Knife® Perfexion, which led to normalization of thyroid hormone secretion and disappearance of the pituitary lesion, as shown in post-radiation MRI. The patient did not experience any side effects from this treatment.

To date, 5.5 years after radiosurgery (October 2015), the patient has remained euthyroid without any treatment (TSH=3.76mIU/L, n.v. 0.3-4.2, FT<sub>4</sub>=0.94 ng/dl, n.v. 0.8-2 and FT<sub>3</sub>= 3.30 pg/ml, n.v. 2-4.4) and without experiencing any short-term or long-term complications. Recent MRI visualization, performed in 2013, showed no evidence of the pituitary adenoma (Figure 3).

Figure 1. Coronal (a) and sagittal (b) MRI images of the pituitary gland, revealing a pituitary adenoma, 9 mm in diameter at diagnosis (February 2002).

Figure 2. Coronal (a) and sagittal (b) MRI images of the pituitary gland, prior to transsphenoidal adenomectomy (April 2006) visualizing a pituitary adenoma, 9 mm at its maximum diameter. The images were taken after failure of treatment with lanreotide for 2 years.
TSHomas treated by gamma knife radiosurgery

DISCUSSION AND REVIEW OF THE LITERATURE

We present a case of TSHoma successfully treated with gamma knife surgery after failure of conventional surgery and escape from somatostatin analog adjuvant therapy. The gold standard of TSHomas treatment is surgical removal of the tumor. Recent data from a retrospective study published by Gatto et al report a post-surgical complications rate of 60% as regards TSHomas, while the same figure is only 12% in the case of GHomas matched for age, sex and tumor size. In our presented case, surgical adenomectomy was performed without any complication but resulted in incomplete removal leaving a residual mass of 3 mm in diameter, and recurrence occurred a little over a year postoperatively.

When surgery fails to cure, as in this case, or if it is contraindicated, medical treatment with somatostatin analogs can be implemented. Two cases of TSHomas effectively treated with SSA monotherapy have recently been published. Most TSHomas express somatostatin receptors and are considered highly sensitive to SSAs. However, common side effects of these drugs, such as gastrointestinal disturbances, cholelithiasis and glucose intolerance as well as resistance to treatment and the TSH escape phenomenon, often contribute to treatment failure. Escape from efficacious treatment was the reason for discontinuing it in this case. Alternative treatment employed in cases of recurrence has been stereotactic irradiation. Stereotactic irradiation of pituitary adenomas is mainly performed with the use of the gamma knife radiosurgery method. A recent review of published studies conducted from 2000 to 2014 on the long-term efficacy and safety of GK treatment for either non-functioning or GH-, ACTH- and PRL-secreting pituitary adenomas treated mainly with GK radiosurgery by Minniti et al indicates excellent long-term tumor control (85-100%), long-term biochemical remission in about 50% of the cases, no significant visual complications and a relatively low (15-35%) incidence of late radiation-induced hypopituitarism. We retrieved 14 published cases of TSHomas treated by gamma knife radiosurgery (Table 1). However, due to the rarity of the tumors, data on the long-term efficacy and safety of the method in the case of TSHomas are anecdotal.

In many of these 14 cases of TSHomas treated by gamma knife, information regarding tumor size, grade of extrasellar invasiveness and irradiation is missing. Wherever it is available, it is striking that by the time of diagnosis most of the tumors presented extrasellar extension and in terms of size they were...
Table 1. Reported cases of TSHomas treated by gamma knife radiosurgery

<table>
<thead>
<tr>
<th>Reference</th>
<th>N, sex</th>
<th>Age (years)</th>
<th>Size</th>
<th>Treatment</th>
<th>Follow-up</th>
<th>Outcome (Thyroid function)</th>
<th>Outcome (MRI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brucker-Davis, 1999</td>
<td>1, F</td>
<td>63</td>
<td>macro</td>
<td>TSS, GK</td>
<td>1 year</td>
<td>Euthyroid</td>
<td>Residual macrotumor</td>
</tr>
<tr>
<td>Losa, 1999</td>
<td>3, NA</td>
<td>NA</td>
<td>macro</td>
<td>TSS, GK</td>
<td>Up to 2 years</td>
<td>Euthyroid, Hypothyroid</td>
<td>2 Reduction in size, Stable</td>
</tr>
<tr>
<td>Ohki, 1999</td>
<td>1, F</td>
<td>64</td>
<td>macro-invasive</td>
<td>TSS, GK</td>
<td>16 months</td>
<td>Euthyroid</td>
<td>NA</td>
</tr>
<tr>
<td>Kon, 2001</td>
<td>1, M</td>
<td>52</td>
<td>macro-invasive</td>
<td>TSS, SSA, cabergoline, repeated TSS, GK</td>
<td>7 months</td>
<td>Euthyroid</td>
<td>NA</td>
</tr>
<tr>
<td>Socin, 2003</td>
<td>1, NA</td>
<td>NA</td>
<td>NA</td>
<td>TSS, GK irradiation</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Clarke, 2008</td>
<td>1, NA</td>
<td>NA</td>
<td>NA</td>
<td>TSS, GK</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Kasliwal, 2012</td>
<td>1, M</td>
<td>24</td>
<td>NA</td>
<td>Preoperative stabilization with SSA, TSS, GK</td>
<td>1 year</td>
<td>Euthyroid</td>
<td>Reduction in size</td>
</tr>
<tr>
<td>Zhao, 2012</td>
<td>4, M</td>
<td>57, 17, 50, 48</td>
<td>1 micro, 2 giant-invasive, 1 macro-invasive</td>
<td>1GK alone, 3SSA, TSS, GK</td>
<td>3 years, 2 years, 1 year, 4 months</td>
<td>Euthyroid</td>
<td>Residual tumor</td>
</tr>
<tr>
<td>Present case, 2015</td>
<td>1, F</td>
<td>43</td>
<td>micro-invasive</td>
<td>SSA, TSS, GK</td>
<td>5.5 years</td>
<td>Euthyroid</td>
<td>No evidence of adenoma</td>
</tr>
</tbody>
</table>

M: male; F: female; TSS: transsphenoidal surgery; GK: gamma-knife; MRI: magnetic resonance imaging; NA: not available; SSA: somatostatin analogs.

macroadenomas (≥1 cm at their maximum diameter). In two cases reported by Zhao et al, the TSHoma was giant-sized, measuring more than 4 cm at its maximum diameter, whereas in one case reported by the same researchers the TSHoma was a microadenoma. The latter is the only case reported in the literature as having been treated by GK radiosurgery alone. In the rest of the cases, as well as in our case, gamma knife radiosurgery was performed as a second-line treatment on the residual tumor after transsphenoidal adenomectomy. In some of the reported cases, medical treatment with SSA was implemented for a short period preoperatively to induce biochemical control and tumor shrinkage and thereby ensure better postoperative results. In the presented case, the use of SSA was extended for a much longer period of time because of the patient’s refusal to undergo transsphenoidal surgery as a primary treatment. In one case reported by Kon et al, SSA treatment was applied for a year after the implementation of GK radiosurgery in order to control the disease until the radiosurgery effect became fully apparent. This might be a useful practice, as a recently published retrospective study on a series of pituitary adenomas by Mak et al indicated that statistically significant tumor size reduction occurs not earlier than 1 year after GK radiosurgery administration. Further reduction, to a lesser extent, continues during the following years as well. A recent review and meta-analysis by Chen, Li et al carried out on a series of non-functioning pituitary adenomas indicated that tumor volume has a statistically significant predictive value of GK effectiveness and safety. Tumor volumes under 4 ml were associated with better tumor control and fewer
radiation-induced complications. This knowledge could also be applicable to TSH tumors. It should be mentioned that in spite of the fact that most TSHomas studied were sizeable by the time of diagnosis, the majority of them received irradiation after surgical removal, when the residual mass was much smaller, probably resulting in higher post-radiation control rates. Another important predictive marker of GK safety is the radiation dose. In order to avoid hypopituitarism, Marek et al highlighted in their study the need to keep the mean radiation dose to pituitary tissue surrounding the adenoma under 15 Gy and the maximum dose to the distal infundibulum under 17 Gy. Similarly, in their review Leavitt et al indicated that the maximum radiation dose to the anterior visual pathway should be kept under 12 Gy in order to avoid radiation-induced optic neuropathy. In the cases discussed here there is no information available on the delivered irradiation dose.

The follow-up in most of the reported cases was minimal, lasting from 4 months to 3 years, and only limited information was provided on the long-term efficacy and safety of the procedure. However, it is striking that in all the above cases where outcome information is available, biochemical remission and tumor control have been achieved without any reported complications. In the presented case follow-up has attained 5.5 years, the longest reported after the use of gamma knife treatment for a TSHoma, without the presence of residual tumor in the post-radiation MRIs performed.

Although there is limited available scientific evidence, on the basis of the favorable results of the previously reported cases of TSHomas treated with GK radiosurgery as well as of the long-term satisfactory outcome of the present case, we suggest that GK radiosurgery should in the future play a more prominent important role in the treatment of recurrent TSHomas. However, more studies based on larger series of patients and longer follow-up need to be conducted to corroborate our present suggestion.

FUNDING

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

DECLARATION OF INTEREST

The authors declare that they have no conflict of interest.

INFORMED CONSENT

Informed consent was obtained from all individual participants included in the study.

REFERENCES

Identification of a novel mutation of the PRKAR1A gene in a patient with Carney complex with significant osteoporosis and recurrent fractures

Labrini Papanastasiou,1 Stelios Fountoulakis,1 Nikos Voulgaris,1 Theodora Kounadi,1 Theodosia Choreftaki,2 Akrivi Kostopoulou,2 George Zografos,3 Charalampos Lyssikatos,4 Constantine A. Stratakis,4 George Piaditis1

1Department of Endocrinology and Diabetes Center, 2Department of Pathology, 3Department of Surgery; “G. Gennimatas” General Hospital, Athens, Greece; 4Section on Endocrinology & Genetics, Program on Developmental Endocrinology & Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, USA

ABSTRACT
OBJECTIVE: Carney complex (CNC) is a rare autosomal dominant multiple neoplasia syndrome characterized by the presence of endocrine and non-endocrine tumors. More than 125 different germline mutations of the protein Kinase A type 1-α regulatory subunit (PRKAR1A) gene have been reported. We present a novel PRKAR1A gene germline mutation in a patient with severe osteoporosis and recurrent vertebral fractures. DESIGN: Clinical case report. CASE REPORT: A 53-year-old male with a medical history of surgically removed recurrent cardiac myxomas was evaluated for repeated low-pressure vertebral fractures and severe osteoporosis. Physical examination revealed spotty skin pigmentation of the lower extremities and papules in the nuchal and thoracic region. The presence of hypercortisolism due to micronodular adrenal disease and the history of cardiac myxomas suggested the diagnosis of CNC; the patient underwent detailed imaging investigation and genetic testing. METHODS: Standard imaging and clinical testing; DNA was sequenced by the Sanger method. RESULTS: Sequence analysis from peripheral lymphocytes DNA revealed a novel heterozygous point mutation at codon 172 of exon 2 (c.172G>t) of the PRKAR1A gene, resulting in early termination of the PRKAR1A transcript [p.Glu58Ter (E58X)]. CONCLUSION: We report a novel point mutation of the PRKAR1A gene in a patient with CNC who presented with significant osteoporosis and fractures. Low bone mineral density along with recurrent myxomas should point to the diagnosis of CNC.

Key words: Carney complex, Hypercortisolism, Osteoporosis, PRKAR1A gene, PPNAD

INTRODUCTION
Carney complex (CNC) is a rare disease with an autosomal dominant inheritance characterized by the presence of myxomas, spotty skin pigmentation and endocrine overactivity.1 CNC is associated with a
variety of endocrine and non-endocrine abnormalities, the most frequent being primary pigmented nodular adrenocortical disease (PPNAD) that usually leads to adrenocorticotropic hormone (ACTH)-independent Cushing’s syndrome (CS). Other common endocrine abnormalities include cystic or nodular thyroid disease in about 75% of patients, subtle hyperprolactinemia (64%), acromegaly (in up to 15%) and large-cell calcifying Sertoli tumors (41% of affected males). Non-endocrine abnormalities include skin myxomas (80% of patients), lentiginosis (80%), breast fibroadenomas or myxomas (50%), cardiac myxomas (20-40%), cutaneous myxomas (20-30%) and psammomatous melanotic schwannomas (8%).

CNC is a genetically heterogeneous disease, with linkage analysis so far identifying two independent loci [17q22-24 (CNC1), 2p16 (CNC2)]; a third locus was erroneously linked to CNC, reflecting the oddity of a single family with myosin mutations and the concurrent occurrence of myxomas.3-5 The most common genetic cause of CNC is a defect in the PRKAR1A gene (at the CNC1 locus). PRKAR1A encodes for the 1-α regulatory subunit (RI-α) of protein Kinase A (PKA) and functions as a tumor suppressor gene (TSG).6 Heterozygous inactivating PRKAR1A mutations lead to CNC with a penetrance close to 98% by the age of 50 years; these mutations have been reported in 73% of CNC patients.2,7,8 The majority of these mutations result in frame-shift, nonsense or splice site variants that lead to premature stop-codon generation.9 Mutant mRNA is unstable and degraded by nonsense-mediated mRNA decay (NMD). This leads to the loss of the mutant protein and a 50% reduction of the total RI-α protein levels, since only the wild type allele is translated.6 RI-α protein reduction stimulates protein kinase A (PKA) activity by cyclic adenosine monophosphate (cAMP) thus interfering in the regulation of cell glucose and lipid metabolism pathways.2 Until recently, about 750 CNC patients have been diagnosed worldwide by the National Institute of Health, the Mayo Clinic (U.S.A), the Cochin Hospital (France) and elsewhere, with more than 125 PRKAR1A gene mutations identified to date (online database: http://prkar1a.nichd.nih.gov).3 Despite the genetic heterogeneity and the large number of PRKAR1A mutations spread along the length of the gene, no direct correlation between all PRKAR1A mutations and the various CNC phenotypes has yet been established. However, recent data report potential associations between specific mutations and CNC manifestations.2,10-13

In this article we present a CNC case with a novel germ line PRKAR1A mutation that was diagnosed after recurrent vertebral fractures presumably due to hypercortisolemia and we provide a brief review of the existing literature concerning genotype and phenotype associations in CNC.

**CASE REPORT**

A 53-year-old male (weight 69 kg, height 1.72 m, blood pressure 110/70 mmHg) was admitted to our Department for evaluation of deteriorating severe osteoporosis (left femoral neck T-score: -4.2, right femoral neck T-score: -4.0, L2-L4 T-score: -5.2), resulting in low-pressure fractures of lumbar spine vertebrae and muscle weakness. Radiologic imaging revealed T8-T10 and L3-L5 vertebral compression fractures. The patient had a history of prior T12-L2 spondylodensis due to a low-pressure L1 vertebra osteoporotic fracture and was treated with denosumab and calcium (500mg bd) for two years without any bone mass improvement. Over the last three decades he had undergone three cardiac operations for recurrent atrial and ventricular peduncular myxomas (left atrium and right ventricle), with his last serial echocardiography not revealing the presence of any myxoma. He had also undergone polipectomy following two episodes of intestinal blood loss. No family history of endocrine or non-endocrine tumors was identified.

On admission he had muscle weakness and incapacitating back pain. Physical examination revealed spotty skin pigmentation (lentigines) on both legs and two 2 cm brownish papules on the preauricular and lower right thoracic region, consistent with cutaneous myxomas. Hormonal work-up revealed hypercortisolism ([08.00 morning cortisol levels: 389 nmol/L (NR: 138-690 nmol/L), adrenocorticotropic hormone (ACTH): 5.5 pg/ml (NR: 9-52 pg/ml), urinary cortisol concentration (UFC): 210 μg/24h (NR: 20-90 μg/24h), cortisol levels following a low-dose dexamethasone suppression test (LDDST): 412 nmol/L and paradoxical increase of UFC (303 μg/24h) following dexamethasone administration]. Secondary
A novel PRKAR1A mutation in a patient with CNC

hyperparathyroidism [parathyroid hormone (PTH): 140 pg/ml (NR: 11-62 pg/ml), corrected plasma calcium: 8.6 mg/dl, plasma phosphorus: 3 mg/dl, 25-OHD3: 15 ng/ml] was also evident. Prolactin [(PRL: 13.9 ng/ml (NR: 3.46-19.4)], free thyroxin [(FT4: 14.8 pmol/L (NR: 9.01-21 pmol/L)], thyrotropin [(TSH: 1.6 μIU/ml (NR: 0.35-4.94μIU/ml)], LH (4.7 mU/ml) and SHBG levels [38nmol/L (NR: 30-100 nmol/L)] were normal. Growth hormone (GH: 2.9 ng/ml) and GH response to a 75gr oral glucose load (nadir of GH: 1 ng/ml) were also normal. Testosterone [1.7 ng/ml (NR: 2.67-10.12 ng/ml)], DHEA-S [468 ng/ml (NR: 518-4707 ng/ml)] and insulin-like growth factor-1 (IGF-1) levels [117 ng/ml (NR for sex and age: 180-406 ng/ml)] were decreased.

Adrenal computed tomography (CT) showed micronodular adrenal disease (Figure 1). Ultrasonography demonstrated multiple thyroid nodules and multiple microcalcifications of the testes, whereas echocardiography did not reveal any recurrent cardiac myxomas. Pituitary magnetic resonance imaging (MRI) was also normal.

Denosumab was discontinued and the patient was prescribed ketoconazole (400mg/day) and metopyrone (1.5gr/day) while on the waiting list for bilateral adrenalectomy. Surgically removed adrenal glands appeared with multiple pigmented nodules (Figure 2) and histopathology revealed multiple, 0.1-0.5cm sized, pigmented nodules surrounded by atrophic cortex (Figure 3). Histological results were consistent with the diagnosis of primary pigmented nodular adrenocortical disease (PPNAD). Most of the nodule cells were large and globular with granular eosinophilic cytoplasm that included lipofuscin. Myxomatous areas were also detected within the nodules (Figure 3).

**Figure 1.** Adrenal computed tomography revealing bilateral nodularity of the adrenal glands.

**Figure 2.** Macroscopic appearance of the surgically removed PPNAD adrenal gland. Multiple pigmented micronodules can be seen in the cross-section.
A peripheral blood sample was drawn after obtaining informed consent from the patient. DNA was extracted from peripheral leucocytes and subjected to polymerase chain reaction (PCR), followed by bidirectional DNA sequence analysis of the \textit{PRKAR1A} gene, performed as described previously.6,14 The nucleotide sequence was compared with the published cDNA \textit{PRKAR1A} sequence. A novel c.172G>T heterozygous point mutation at codon 172 of exon 2 was identified that resulted in direct stop-codon generation and in early termination of \textit{PRKAR1A} transcript (Figure 4).

**DISCUSSION**

CNC is a rare endocrine syndrome which is most frequently caused by \textit{PRKAR1A} gene mutations. We report a novel germline nonsense mutation of the \textit{PRKAR1A} gene in a patient with clinical features as well as laboratory and histological findings of CNC. Our patient had developed a wide heterogeneous spectrum of CNC-associated manifestations including hypercortisolism with PPNAD and secondary osteoporosis, skin lentigines and myxomas, multiple recurrent cardiac myxomas and nodular thyroid disease. We also found a paradoxical increase of cortisol secretion after dexamethasone administration, which is characteristic of CNC and particularly useful for the diagnosis of patients with normal baseline cortisol levels and subclinical or cyclic CS.15

Osteoporotic bone changes are often found in CNC patients. Glucocorticoids excess accelerates bone resorption and reduces bone formation by direct action.
A novel *PRKAR1A* mutation in a patient with CNC

In addition, glucocorticoids decrease intestinal calcium absorption by opposing the action of vitamin D and by decreasing the expression of calcium channels in the duodenum leading to secondary hyperparathyroidism. They also inhibit IGF-1 and serum testosterone production (as observed in our patient). Apart from the secondary osteoporosis due to PPNAD-associated cortisol hypersecretion, the osteogenic potential may also be influenced by *PRKAR1A* gene ablation that can interfere with signaling pathways which are necessary for osteoblast differentiation, as shown in experimental data from mouse and human cell lines. The osteoprotic bone changes, fractures and secondary hyperparathyroidism that were found in our CNC patient indicated the long duration of the disease.

Recurrent cardiac myxomas occur at a much younger age in CNC patients compared to sporadically occurring myxomas. Interestingly, up to 30% of non-CNC myxomas can also bear *PRKAR1A* muta-
tal delay. Moreover, the average age of presentation was younger (14 years) and morbidity more severe than in typical PRKAR1A mutation-positive CNC patients. In addition, in a family of CNC patients, a spectrum of disease including adrenal carcinoma due to a S147G point mutation in the PRKAR1A gene that escapes NMD, was identified. This mutation led to decreased cAMP and catalytic subunit binding by R I-α and increased PKA activity in vitro. 11

Lately, genes coding for other PKA subunits have been identified as being responsible for CNC-linked phenotypes (but not the full syndrome). Duplication of the main catalytic subunit of the cAMP-dependent PKA, Cα (PRKACA), may result in Cushing syndrome caused by bilateral micronodular adrenal hyperplasia. 12 In a single patient, triplication of the catalytic subunit Cβ (the PRKACB gene) was associated with skin pigmentation, acromegaly and myxomas. 13

All the aforementioned genetic data could have implications for our understanding of the CNC phenotype and, more importantly, for counseling CNC patients. Genetic analysis is a powerful tool aiding the investigation, confirmation and early identification of tumors and endocrine disorders related to CNC. Younger age of onset and potential diverse prognosis according to the genotype could help us properly counsel patients’ family members and descendants. Even though there is yet no obvious and straightforward correlation between genotype findings and the clinical characteristics of the disease, there is growing evidence that a genotype-phenotype association is plausible for at least certain PRKAR1A defects. 21,22 Two PRKAR1A mutations (a deletion c.709-7del6 and a mutation in the initiation codon of PRKAR1A, M1V c.1A>G/p.M1Vsubstitution) are associated with low-penetrance, early life isolated PPNAD and CS. 23,24 Moreover, in some cases of isolated PPNAD, a small intronic deletion in PRKAR1A has been associated with a lower penetrance and mild phenotype. 25 In general, patients with exonic PRKAR1A mutations seem to present at a younger age and to manifest cardiac myxomas, lentigines, schwannomas and acromegaly more often compared to patients with intronic ones.

In the study of Salpea et al, a significant number (21.6%) of CNC patients had haploinsufficiency due to large 17q24.2-q24.3 deletions surrounding the PRKAR1A gene. 10 These deletions were not detected by Sanger sequencing (PRKAR1A mutation-negative) but with array-based comparative genomic hybridization (array-CGH). It is worth noting that apart from the usual CNC-related manifestations, some of these patients shared skeletal abnormalities and global developmental delay. Moreover, the average age of presentation was younger (14 years) and morbidity more severe than in typical PRKAR1A mutation-positive CNC patients. In addition, in a family of CNC patients, a spectrum of disease including adrenal carcinoma due to a S147G point mutation in the PRKAR1A gene that escapes NMD, was identified. This mutation led to decreased cAMP and catalytic subunit binding by R I-α and increased PKA activity in vitro. 11

In contrast to RET mutations in MEN2 syndrome, it is widely accepted that PRKAR1A gene mutations do not appear to correlate consistently with a specific clinical phenotype. However, data overall indicate that CNC patients bearing PRKAR1A mutations (CNC1) have more severe disease with earlier presentation and higher frequency of myxomas, thyroid and gonadal tumors, schwannomas and lentigines compared to PRKAR1A negative patients with a mutation mapped in the 2p16, CNC2 locus. 5,4,14 In addition, a small number of PRKAR1A missense mutations whose mRNA escape from NMD and express R I-α mutant proteins are associated with a more severe phenotype. 21,22 Two PRKAR1A mutations (a deletion c.709-7del6 and a mutation in the initiation codon of PRKAR1A, M1V c.1A>G/p.M1Vsubstitution) are associated with low-penetrance, early life isolated PPNAD and CS. 23,24 Moreover, in some cases of isolated PPNAD, a small intronic deletion in PRKAR1A has been associated with a lower penetrance and mild phenotype. 25 In general, patients with exonic PRKAR1A mutations seem to present at a younger age and to manifest cardiac myxomas, lentigines, schwannomas and acromegaly more often compared to patients with intronic ones.

In conclusion, we have identified a novel PRKAR1A nonsense mutation in a sporadic case of CNC. The data add to what we know about PRKAR1A and human disease. Recognizing CNC is important for identifying early and addressing promptly the many associated comorbidities, including acromegaly, atrial fibrillation and stroke due to atrial myxomas, diabetes mellitus, hypertension, osteoporosis and fractures due to hypercortisolism, and the risk of various tumors and certain cancers. PRKAR1A mutation analysis should be undertaken in suspected cases of CNC in order to confirm the diagnosis and provide close monitoring and follow-up.
ACKNOWLEDGEMENTS/FUNDING

This work was supported by the Intramural Research Program (IRP) of the Eunice Kennedy Shriver National Institute of Child Health & Human Development (NICHD), National Institutes of Health (NIH), Bethesda, MD 20892, USA.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to disclose.

REFERENCES

Sir,

The last 100 years have seen a revolution in the understanding of adrenal disease and its surgical treatment. The isolation of its hormones, the detailed study of the adrenal medulla and the cortex together with the enormous expansion of surgical methods served as catalysts to this revolution. The Greek word for adrenal (epinephridio, from the Greek epi, upon, and nephros, kidney) dates back to the age of Homer who mentions the adrenal glands. In his Iliad (21, 204), when describing Achilles’ killing of an adversary, Homer writes: “About him, indeed, the eels and fishes were busied, eating and nibbling the fat around his kidneys”.1 Although Homer seems to be aware of the tissue upon the kidneys, translated by Buckley as fat,1 and because of his extensive anatomical knowledge, he appears here to be referring to the left flank in general. In the Bible, Leviticus (3:4) contains a similar description of the adrenals, which again were understood to be integral parts of the kidneys: “the kidneys and the fat that is on them at the loins”.2

Galen was the first to provide a detailed description of the adrenal veins, defining the adrenal glands as “bodies around the kidneys”: “For when the vein first appears outside the liver, before reaching the loin, being still high, at its own right side, it sends to the capsule of the right kidney and the bodies [suprarenals?] around this sometimes spiderweaver-like, sometimes hairlike, and sometimes thicker contributions”3,4

Bartolomeo Eustachio (or Bartholomeus Eustachius), a famous Italian anatomist who flourished in 16th century Rome, was the first to recognize the adrenal gland as a distinct anatomical entity. His treatise titled “Opuscula Anatomica” and published in Venice in 1564 contains the first description of the adrenal glands, which he labeled “glandulae quae renibus incumbent” (glands lying on the kidney).5 He defines the adrenals as glands “diligently overlooked by other anatomists”, that are capped on the upper kidney surface resembling a kidney in shape but sometimes bigger in size: “Both kidneys are capped on the extremity towards the cava by a gland. Both are connected with a fold of the peritoneum in such a way that one, if he is not very attentive, does really overlook them, as if they were not present. Their shape resembles that of the kidneys...sometimes one is bigger, sometimes another...early anatomists and those who write ample treatises on this art in our days failed to

Key words: Adrenalectomy, Adrenal glands, Adrenal surgery, Endocrine surgery, Cushing disease, Pheochromocytoma

Address for correspondence:
Marios Papadakis, Department of History of Medicine, School of Medicine, University of Crete, Voutes Campus, Heraklion, Crete, GR-71003, Greece;
E-mail: mpapadakis@med.uoc.gr
Received 06-07-2015, Accepted 16-07-2015
detect them”\(^6,7\). Assisted by Pier Matteo Pini, an artist relative, Eustachio prepared an excellent series of copperplate engravings of anatomical illustrations. The series included the first depiction of the adrenal glands (Figure 1) and was meant to accompany a medical treatise, left however uncompleted when Eustachio died in 1574. The engravings passed through Pini and his heirs into the Vatican Library’s possession where they remained unprinted until discovered by Giovanni Maria Lancisi at the beginning of the 18th century. Lancisi, personal physician to Pope Clement XI, published the entire series of 47 splendid plates under the title: “Tabulae Anatomicae Bartholomei Eustachi quas a tenebris tandem vindicatas” (Anatomical Illustrations of Bartholomeo Eustachio rescued from obscurity) in 1714.

Eustachius’ original description of the adrenals provided a new field for argument with his contemporaries over the ensuing years. Arcangelo Piccolomini (1562-1605) in his treatise\(^8\) vented bitter criticism of Eustachius’ findings, reporting that the adrenal glands do not deserve special attention, as they represent rare renal excrescences, caused in a way similar to that causing supernumerary digits:\(^6,7\) The fact that the glands were rarely identified was underlined by André Du Laurens, physician to Henry IV of France, who in 1640 wrote: “Eustachius claims to find a gland above the kidneys. Sometimes we saw that too; often, however, we stated that there was no such gland”\(^6\).

In 1627, Casserius, a prominent anatomist of Padua, validated Eustachius’ discovery and provided illustrations of the adrenal glands, which he termed corpuscula reni incumbientia sive renes succenturiati (renal corpuscles lying on or above the kidney).\(^9\) Casserius’ depiction (Figure 2) was the first to appear, as Eustachius’ plates were not published until two centuries after being engraved. His work, however, did not receive the attention it deserved. Marcus Aurelius Severinus (1580-1658) is thought to have been the first to mention a relation between the left adrenal gland and epididymis through an excretory duct. This discovery, suggestive of an “adrenogonadal relationship”, was largely overlooked and attributed to Antonio Maria Valsalva (1666-1723) in 1719.\(^6\)

The name, suprarenal gland was introduced by Riolan the Younger in 1655\(^6\) and the “modern” terms medulla and cortex were introduced in 1836 by Nagel.\(^10\) By the early 18\(^{th}\) century, medicine had over 20 theories seeking to explain the function and physiology of the adrenal glands, whose presence was by this time well-established. A comprehensive overview of all these theories is beyond the scope of this study. Casper Bartholin, who considered the adrenals to be hollow organs filled with black bile, called them capsulae atrabiliarae, while an important milestone was reached in 1636 by Thomas Wharton. Wharton was the first to associate the adrenal glands with the nervous system, suggesting a possible role for them in transferring substances from the nerves to the

---

Figure 1. The first depiction of the adrenal glands available by Eustachius in 1552. (Reproduction from: Eustachi B, Lancisi GM. Tabulae anatomicae clarissimi viri Bartholomaei Eustachii quas è tenebris tandem vindicatas: Edition Medicina Rara; 1714. In public domain).
circulation. His idea was affirmed 200 years later when Kölliker provided the first accurate description of the microscopic anatomy of the adrenal gland in 1852. In 1716, the Academy of Sciences of Bordeaux held a competition offering a prize for an answer to the question “What is the function of the suprarenal glands?” The then 29-year-old French philosopher Montesquieu was asked to judge the competition. However, finding no contestant convincing enough to be awarded the prize, he expressed the hope that the problem would someday be solved. In 1805, Cuvier established that the adrenals were solid structures and in 1894 George Oliver and Edward Albert Schäfer were the first to demonstrate a hormonal effect by establishing a relation between adrenal medulla and blood pressure. Concluding their findings in a treatise published in 1895, they clarify “that one of the main functions, if not the main function, of the suprarenal capsules is to produce a material which is added in some way or another to the blood.”

In 1855, Thomas Addison, in his final publication before his death titled “On the constitutional and local effects of disease of the ‘suprarenal capsules’”, reported eleven patients with various symptoms, as “anaemia, general langour and debility, remarkable feebleness of the heart’s action, irritability of the stomach and a peculiar change of colour in the skin, occurring in connexion with a diseased condition of the ‘suprarenal capsules’”. Addison states that “the disease is by no means of very rare occurrence.” In 1896, William Osler first used an adrenal extract to treat a 21-year-old girl suffering from Addison disease who died “during the treatment with the suprarenal extract”. Osler, however, kept up his research and in the same year published his experience with 6 addisonian patients, including one who greatly benefited from the use of the extract. In 1897, John Jacob Abel became the first to isolate an endocrine secretion as a chemically-pure substance, i.e. epinephrine. Some years later Jokichi Takamine and Thomas Bell Aldrich Takamine were the first to independently isolate epinephrine in a crystalline form. The first description of a successful adrenalectomy was reported in 1890 by Thornton, who one year earlier employed a T-shaped incision to remove a 20-pound adrenal tumor with the left kidney from a 36-year-old woman. The tumor was most probably a malignant adrenocortical carcinoma and the patient died 2 years later from recurrence. In 1932, Lennox Broster described a unilateral, transpleural, transdiaphragmatic adrenalectomy through a long posterior, intercostal approach for adrenogenital syndrome associated with cortical hyperplasia. In the same year, Cushing was the first to recognize the role of the adrenal cortex in pituitary baseophilia, describing an endocrine syndrome causing hypercortisolism through excessive secretion by the cortex. The disease, now referred to as Cushing syndrome, was termed “suprarenal cortical syndrome”, a term also sometimes used to describe adrenal virilism. By this time, the search for potent cortical extracts had reached its peak. The research was primarily led by Edward Calvin Kendall at the Mayo Clinic and Reichstein in Zürich. Their intensive efforts resulted in the isolation of cortisone
and its essential components in the late 1930s. In 1934, Walters et al at the Mayo Clinic were the first to report ten cases of subtotal adrenalectomy for patients with “suprarenal cortical syndrome”, with a mortality rate of 30% despite using Kendall’s potent cortical extracts. In 1936, Hugh Young of Johns Hopkins reported a posterior approach with the removal of the 12th rib for simultaneous exposure of both adrenals in Cushing patients.

In 1949, James Priestley at the Mayo Clinic reported his experience in 29 adrenalectomized patients with Cushing syndrome. In the first 20 cases, a pre- and postoperative replacement therapy with aqueous adrenal cortical extract was employed. The last nine cases were treated with cortisone in place of aqueous adrenal cortical extract. No deaths were observed in the cortisone group, in comparison with the 30% mortality rate in the aqueous cortical extract group. These results, supported by larger subsequent studies, established the use of the replacement therapy. In 1886, Felix Fränkel described an 18-year-old woman who died from progressive circulatory failure 9 days after hospital admission with paroxysmal hypertensive crises. The autopsy revealed bilateral adrenal tumors, which apparently were pheochromocytomas. Since no signs of Addison’s disease, the only known clinical entity of adrenal pathology at the time, were observed, Fränkel attributed the death to severe nephritis, considering the adrenal tumors as latent. Similar adrenal tumors were identified in subsequent years and were termed “paragangliomas”, coined by Alezais and Peyron in 1908. It was not until 1912 that the term pheochromocytoma was used by Ludwig Pick to refer to tumors in the adrenals and at extra-adrenal sites. The first comprehensive tumor description together with association with symptoms and signs was published 10 years later by Labbé et al. In 2007, Neumann et al reviewed Fränkel’s publication, addressing the hypothesis that the patient had an inherited disorder. The presence of germ-line RET mutations in four living descendants established the clinical and molecular diagnosis of MEN-2 in Fränkel’s original description.

The first surgically treated patient with a pheochromocytoma in 1923 died in shock, the event being regarded as “disproportionate to the severity of the operation”. The first known successful surgical removal of a pheochromocytoma was performed on 25 February 1926 by César Roux (1857-1934) in Lausanne, Switzerland. The patient was a woman suffering from paroxysmal hypertensive crises who had noticed an orange-sized lump under her right costal margin, with an X-ray pattern resembling a liver tumor. The patient underwent surgical exploration and a tumor attached to the right adrenal was found and removed. The postoperative period was uneventful with no recurrence or complications during a follow-up period of 18 months. The second successful removal of a pheochromocytoma was independently carried out by Charles Mayo 7 months later in Minnesota, USA. Using a flank incision, Mayo removed a lemon-sized tumor, apparently separate from the adrenal of a patient with paroxysmal hypertension. The tumor, with the appearance of a typical pheochromocytoma, was given several names, such as “retroperitoneal malignant blastoma” and “encapsulated fibrous cellular retroperitoneal malignant neoplasm”. The patient was disease-free during the follow-up period and died after 18 years from coronary thrombosis. The following year, 1927, Mayo had his work published, one year earlier than Roux, whose case was included in the thesis of Roland von der Mühl, a pathologist working in Lausanne, published in 1928. Thus, Mayo was primarily credited with the first successful pheochromocytoma removal and Roux is best known for the Roux-en-Y anastomosis, popularized in 1893. By 1940, about 20 operations for pheochromocytomas had been reported, a number that rose to 151, with a 26% mortality rate, by 1951.

The evolution of laparoscopic adrenalectomy serves as the first-line of therapy for the management of most functional and non-functional adrenal tumors. Gagner et al were the first to report three cases of laparoscopic adrenalectomy in the lateral decubitus position in two patients with Cushing syndrome and a 60-year-old man with pheochromocytoma in 1992. This technique gained widespread acceptance, being considered the most popular technique for the past decade. In the same year, Higashihara et al published in Japanese a case of laparoscopic adrenalectomy for primary aldosteronism utilizing a subcutaneous steel traction method to reduce CO2 insufflation pressure. The following year, the authors summed up their experience with this method reporting three
patients with primary aldosteronism who underwent successful laparoscopic adrenalectomy. Gagner recognizes two presentations of their findings at the American Association of Endocrine Surgery and the American Surgical Association as landmarks “that gave a stamp of approval for this technique”, stating that “the work of a pioneer is not only to describe the technique but also to convince the world surgical community that these changes are significant”. In the latter of the two works, the authors reported 97 laparoscopic adrenalectomies with no mortality, no recurrence of hormonal excess and a conversion rate of 3%, suggesting that the open procedure should be reserved for cases of invasive adrenal carcinoma and malignant pheochromocytoma. Mercan et al should be credited for introducing the endoscopic retroperitoneal approach in clinical practice, recording successful resection in eight patients with benign adrenal lesions in 1995. Both these innovative surgical techniques simplified the procedure, resulting in less postoperative morbidity and a shorter recovery period, this becoming the new gold standard in adrenal surgery.

REFERENCES

5. Eustachius B 1564 Opuscula anatomica de renum structura, effico et adminstratione, Vicentius Luchinus, Venice.
17. Osler W, 1896 On six cases of Addison’s disease: with the report of a case greatly benefited by the use of the suprarenal extract. Internat Med Mag 5: 3-11.
30. Labbé M, Tinel J, Doumer A, 1922 Crises solaires et hypertension paroxystique en rapport avec une tumeur
34. von der Mühll R 1928 Contribution à l’étude des paraganglions de la surrénale, G. Vaney-Burnier SA.
Does ambient light at night reduce total melatonin production?

Christopher C.M. Kyba,1,2 Thomas Kantermann3,4

1Deutsches GeoForschungsZentrum GFZ, 2Leibniz-Institute of Freshwater Ecology and Inland Fisheries, 3Chronobiology Unit, Groningen Institute for Evolutionary Life Sciences, University of Groningen, Groningen, The Netherlands, 4Institute for Occupational, Social and Environmental Medicine, Clinical Centre Ludwig-Maximilians University Munich, Munich, Germany

Dear Editor,

It was with great interest that we read the recent study by Hersh et al1 on the effects of sleep and light at night on melatonin in adolescents. Of particular interest was their focus on electronic use after “lights out”. The authors highlight the importance of understanding what effects this may have on sleep, citing a survey that showed that 72% of American 13-18 year olds regularly use a cellphone or computer before trying to go to sleep.2 In their study, Hersh et al1 did not observe a significant suppression in urinary morning melatonin (aMT6s) levels with respect to the use of electronic devices between lights off and sleep onset. Therefore, the authors conclude that “nighttime behaviors of adolescents by and large do not impact urinary melatonin levels”. Absence of evidence, however, is not the same as evidence of absence, and we believe that the authors' conclusion is premature.

A casual reader not examining their tables may be surprised to learn that the data of Hersh et al1 in fact are most consistent with a relatively large melatonin reduction associated with the use of electronic devices. Compared to adolescents who used electronic devices for less than one hour after lights off on weekdays, the authors observed a mean reduction in aMT6s of 21% in adolescents who used electronics for 1-2 hours during the same period. Their findings, therefore, are entirely consistent with two opposing hypotheses: First, that electronic use has no effect on melatonin, and second that electronic media are associated with a relatively large decrease in aMT6s. Presenting the conclusions in this way makes a much stronger case for the need for a follow-up study, with an approximately 15 times larger sample size.

Hersh et al1 also demonstrated that subjective assessments of ambient bedroom lighting was poorly correlated with aMT6s. However, the data suggest that such subjective assessments may not be accurate: only 5 participants reported being able to read, despite the fact that 22 said a light or TV was on in or near the bedroom overnight and 45 reported a light outside of the bedroom window. We suggest follow-up studies should add (freely available) nighttime imagery of the participant’s location3 to the study repertoire, combined with questions about the use of sleep masks and/or window shades, and importantly, light exposure history the previous day.4 Chronotype, as the authors point out, is essential to disentangle...
the individual contribution of light exposure on the circadian system.\textsuperscript{5} However, when chronotype is to be used as a reference measure for circadian phase, it is preferable to consider other individual phase markers in addition to using amplitude measures (such as aMT6s here). One example is the dim light melatonin onset, which is highly correlated with chronotype.\textsuperscript{6}

As a final thought, it is worth considering the role of smartphones in future studies.\textsuperscript{7} The iPhone camera, for example, can accurately measure radiance down to starlight levels,\textsuperscript{8} and the gyroscope and accelerometer provide evidence of the participant’s activity. Together, these sensors could provide far more accurate assessment of “in bed”, “lights out”, and “phone off” timing than participant reporting.

FINANCIAL SUPPORT

CCMK is financed by the GFZ German Research Centre for Geosciences. TK is supported by the Technology Foundation STW grant P10-18/12186. CCMK and TK acknowledge the support of EU COST Action ES 1204 for providing the opportunity for in-person discussions.

REFERENCES

8. Dark Sky Meter App: www.darkskymeter.com
Letter to the Editor

Central precocious puberty due to hypothalamic hamartoma in neurofibromatosis type 1

Emanuele Bartolini,1,2 Stefano Stagi,3 Perla Scalini,3 Andrea Bianchi,4 Antonio Ciccarone,5 Mario Mascalchi4

1Neurology Unit and Laboratories, Anna Meyer Children’s University Hospital, Florence; 2Stella Maris Foundation, Calambrone, Pisa; 3Health Sciences Department, University of Florence, Anna Meyer Children’s University Hospital, Florence; 4Quantitative and Functional Neuroradiology Research Program at Anna Meyer Children’s and Careggi University Hospitals, University of Florence, Florence; 5Medical Physics Unit at Anna Meyer Children’s University Hospital, Florence; Italy

Dear Editor,

In neurofibromatosis type 1 (NF1), Central Precocious Puberty (CPP) occurs almost invariably in association with optic pathway tumors, usually pilocytic astrocytoma.1 However, at times the aetiology remains undefined.2 On the other hand, in the general population CPP develops due to an underlying hypothalamic hamartoma in up to 10% of patients.3 To the best of our knowledge, CPP due to hypothalamic hamartomas in NF1 has been reported only once in the literature.4

Herein we report the case of a young girl with NF1 who developed CCP related to a hamartoma of the tuber cinereum at 5 years of age. NF1 was diagnosed at 3 months of age because of the presence of about 30 café-au-lait cutaneous spots (diameter >0.5 mm) associated with iris Lisch nodules. Thereafter she developed axillary and groin freckling and three subcutaneous neurofibromas. Familial clinical history was negative. Precocious puberty was diagnosed at age 5.3 years, with increased growth velocity (from the 25th to the 90th percentile) and thelarche (Tanner breast stage 2). LH-RH test was positive (luteinizing hormone -LH- peak 10.1 mUI/ml). Bone age was correspondent to chronological age, while pelvic ultrasonography revealed increased ovarian volume and a transitional uterus. Brain Magnetic Resonance Imaging (MRI) showed an oval 13x14 mm mass in the tuber cinereum region, isointense to gray matter on all sequences, with no displacement on the third ventricle and no contrast enhancement, consistent with hypothalamic hamartoma.5 In addition, multiple round areas of T2 hyperintensity consistent with unidentified bright objects (UBOs) and not showing contrast enhancement were present in the peridentate white matter, right superior cerebellar peduncle and hippocampus, bilaterally. Treatment with intramuscular Enantone 3.5 mg every 28 days was started with a rapid biochemical and clinical response (after 6 months of therapy, Tanner breast stage was 1, LH peak was 1.4 mUI/ml). Treatment was suspended at the age of 11.3 years. During follow-up the pituitary function was always normal. No seizure occurred.
most importantly of the gelastic type. Serial control MRIs were performed until age 18 and demonstrated no changes of the tuber cinereum lesion (Figure 1) or UBOs. At the last 3T MRI we also performed quantitative proton MRI spectroscopy using an external reference phantom. Single voxel short TE spectra were obtained from the hypothalamic lesion (Figure 1) and from the apparently normal right parietal white matter: this showed a lower concentration (8.4 mmol/kg) of N-acetyl-aspartate (NAA) and a higher concentration (4.6 mmol/kg) of myo-inositol (mI) and choline (Cho) (3.0 mmol/kg) in the former compared to the latter (NAA 13.7 mmol/kg; mI 1.6 mmol/kg; Cho 1.6 mmol/kg), whereas the concentration of creatine was similar (lesion 9.9 mmol/kg; normal WM 10.3 mmol/kg). These quantitative data are in agreement with the typical spectroscopy pattern of a tuber cinereum hamartoma. In particular, increased myo-inositol is assumed to reflect increased glial content, whereas decreased NAA is related to neuronal substitution.

Hypothalamic hamartomas are heterotopic non-neoplastic masses of grey matter composed of neurons and glia cells of normal appearance and structure. They are usually small lesions, measuring between 0.5 and 2 cm in diameter, which as a rule remain unchanged over time. The typical location is in the midline, in proximity to the tuber cinereum and the mammillary bodies.

From the neurobiological point of view, hamartomas are to be distinguished from UBOs which are common in NF1 and typically arise before adolescence and disappear in adult age. UBOs are unlikely to be related to CPP because they are usually localized symmetrically or asymmetrically in the cerebral hemispheres, brainstem and cerebellum. It should be noted, however, that differential diagnosis between hypothalamic hamartoma and optic pathway glioma on MRI and proton MR spectroscopy is based on signal heterogeneity and frequent presence of contrast enhancement areas, as well of lactate and lipids in the MR spectroscopy of optic pathway gliomas.

In conclusion, our observations suggest that CPP can be due to a tuber cinereum hamartoma also in NF1, with diagnostic, prognostic and therapeutic implications that appear analogous to hamartoma-related CPP in the general population. Conversely, the admittedly rare possibility of NF1 has to be entertained in every new diagnosis of hamartoma-related CPP.

DISCLOSURE

The authors report no disclosures relevant to the manuscript.

REFERENCES


Diversity in endocrinology practice: the case of Ramadan

Ioannis Ilias,1 Luai Said Tayeh,1 Isidoros Pachoundakis2

1Department of Endocrinology, Elena Venizelou Hospital; 2Department of Sociology, Panteion University; Athens, Greece

There is today an imperative need to raise health workers’ awareness of contemporary cultural diversity and different beliefs, values and attitudes concerning health and disease in order to provide culturally appropriate and professionally competent care.

Muslims currently account for 1%-10% of the population in Western Europe (http://www.pewforum.org/2011/01/27/tablemuslimpopulationgrowthbycountry). It is obligatory for every Muslim to fast once a year, and specifically during the month of Ramadan.1 Ramadan migrates over the seasons since its dates are based on the lunar calendar. It starts with the New Moon and ends with a three-day celebration called Eid al-Fitr (End of Fasting Feast, i.e. Small Holiday).

During Ramadan all healthy adult Muslims are obliged to abstain from any kind of food, water, chewing gum, all kinds of tobacco, as well as from sex from sunrise to sunset. However, fasting is purely the external and practical part of Ramadan. Spiritual aspects of fasting ban lying, gossiping or deceiving. As defined in the Qur’an, fasting is a strict practice of deep personal worship in which Muslims seek the highest level of awareness of the Divine. Two main meals are included in Ramadan: Iftar after sunset and Suhur before sunrise. From Iftar to Suhur and until the next day everyone can eat what he/she wants, including sweets and drinks, but avoiding alcohol and pork (the last two are strictly forbidden in the Qur’an).

Some people, e.g. the temporarily sick and travelers, are excluded from fasting, but after their treatment or finishing their journey, they must fast in compensation for the days that they have lost. It should be emphasized that the exemption includes women who are pregnant, breastfeeding or menstruating.2 Also exempt for the whole month of Ramadan are those that are suffering serious and/or chronic health problems (e.g. diabetes or cardiovascular disease) but they should feed instead a poor man every day throughout Ramadan.

Ramadan may be challenging from an endocrinologist’s point of view since the fasting individual should remain without food or water/drink for many hours. Furthermore, according to some interpretations, p.o. treatment should be proscribed during daylight in the month of Ramadan.3 Longer acting glucocorticoids (such as prednisolone or dexamethasone) –at least to cover daylight replacement needs– may be considered for patients with adrenal insufficiency shortly before and during Ramadan.3

Key words: Adrenal insufficiency, Diabetes mellitus, Fasting, Islam, Ramadan

Address for correspondence:
Ioannis Ilias, Department of Endocrinology and Diabetes,
Elena Venizelou Hospital, 2 E. Venizelou street,
GR-115 21, Athens, Greece;
Tel.: +309555662573; E-mail: iliiasmmd@yahoo.com
Received:17-11-2015, Accepted: 19-11-2015
Although pregnant women are exempt from fasting, if they do fast they risk developing ketosis. Surprisingly, a small study from Turkey did not concur with this finding.4

Furthermore, although patients with diabetes can also be exempt from fasting, many do fast; hypoglycemia is then a concern since in patients with diabetes mellitus type 2 (DM2) who fast the risk is increased fivefold.5 Patients with diabetes mellitus type 1 who fast risk diabetic ketoacidosis.5 Dehydration is also possible.5 Nevertheless, in patients with well-controlled DM1 or DM2 fasting is feasible.

For patients with DM2 on p.o. treatment caution should be applied in the use of sulfonylureas (SU) as these medications carry an inherent risk of hypoglycemia. For once-daily dosing a SU should be given before Iftar (i.e. the meal after sunset, with appropriate dosage adjustments), while for twice-daily regimens the SU dose before Suhur (i.e. the meal before sunrise) should be halved, keeping unchanged the SU dose before Iftar.2,5 Glitidines should be taken before Iftar and Suhur.5 A DPP4-inhibitor is a sensible p.o. antidiabetic choice for Ramadan with practically no hypoglycemia risk.6

For patients with DM2 on insulin or patients with DM1, a basal-bolus regimen is advised,2,5 taking, however, into account the paucity of relevant data.5 A 20% dosing reduction in long-acting insulin analogues, given with Iftar, has been proposed to reduce the risk of hypoglycemia.7

Ramadan is important for all Muslims and is full of life lessons. Fasting prompts thinking about the lives of the poor and of those without food. It is the month of love, mercy, kindness, charity and union of all Muslims. In the absence of guidelines, individualized preparation and drafting of therapy regimens should be implemented for Muslims with adrenal insufficiency or diabetes who choose to observe the fast of Ramadan.5,8,9

REFERENCES

To the Editor,

The study by Huang et al\textsuperscript{1} sheds valuable light on how obesity may cause depression. However, depressed people are known to be at higher risk for developing obesity in the first place.\textsuperscript{2} It is therefore important to know whether any of the participants had a history of prior depression. Other significant risk factors for depression such as concomitant physical illness, medication use, lack of family support and presence of pain should also be investigated. Depressive symptoms have been especially linked to general medical\textsuperscript{3} and, in particular, to cardiovascular conditions,\textsuperscript{4} which have a higher prevalence in obese individuals. In addition, the Beck Depression Inventory includes questions about physical symptoms such as fatigue, which may result in the scores being higher, since symptoms of illness may be confused with depression. Finally, insulin resistance was defined as a HOMA-IR (homeostasis model of assessment for insulin resistance) score of >1.64, whereas the most widely adopted HOMA-IR cutoff value is is 2.6.\textsuperscript{5}

REFERENCES

Dear Editor,

It is my great pleasure to respond to the letter by Dr. Canbaz.

Our study is based on a subsample of a prospective randomized study (ChiCTR-OCS-12002381). The participants enrolled had no history of prior depression or any other systemic comorbidity, such as cardiovascular disease, medication use, chronic pain, etc. Such symptoms as fatigue may cause the bias of higher scores in BDI-II. It is very difficult to identify whether the fatigue results from depression or physical illness. It is, in any case, only a small component and the impact is minor. In addition, since in this study we aimed to demonstrate that AN is associated with severe depression symptoms in obese patients, the control group of obese patients without AN helped to reduce the bias. As to the HOMA-IR cutoff value, the value of 1.64 was based on Chitturi’s study entitled “NASH and Insulin Resistance: Insulin Hypersecretion and Specific Association with the Insulin Resistance Syndrome” (Hepatology 2002;35:373-379). In their original study, subjects were categorized as insulin resistant if the HOMA-IR value was greater than 1.64.

Sincerely yours,
Yueye Huang