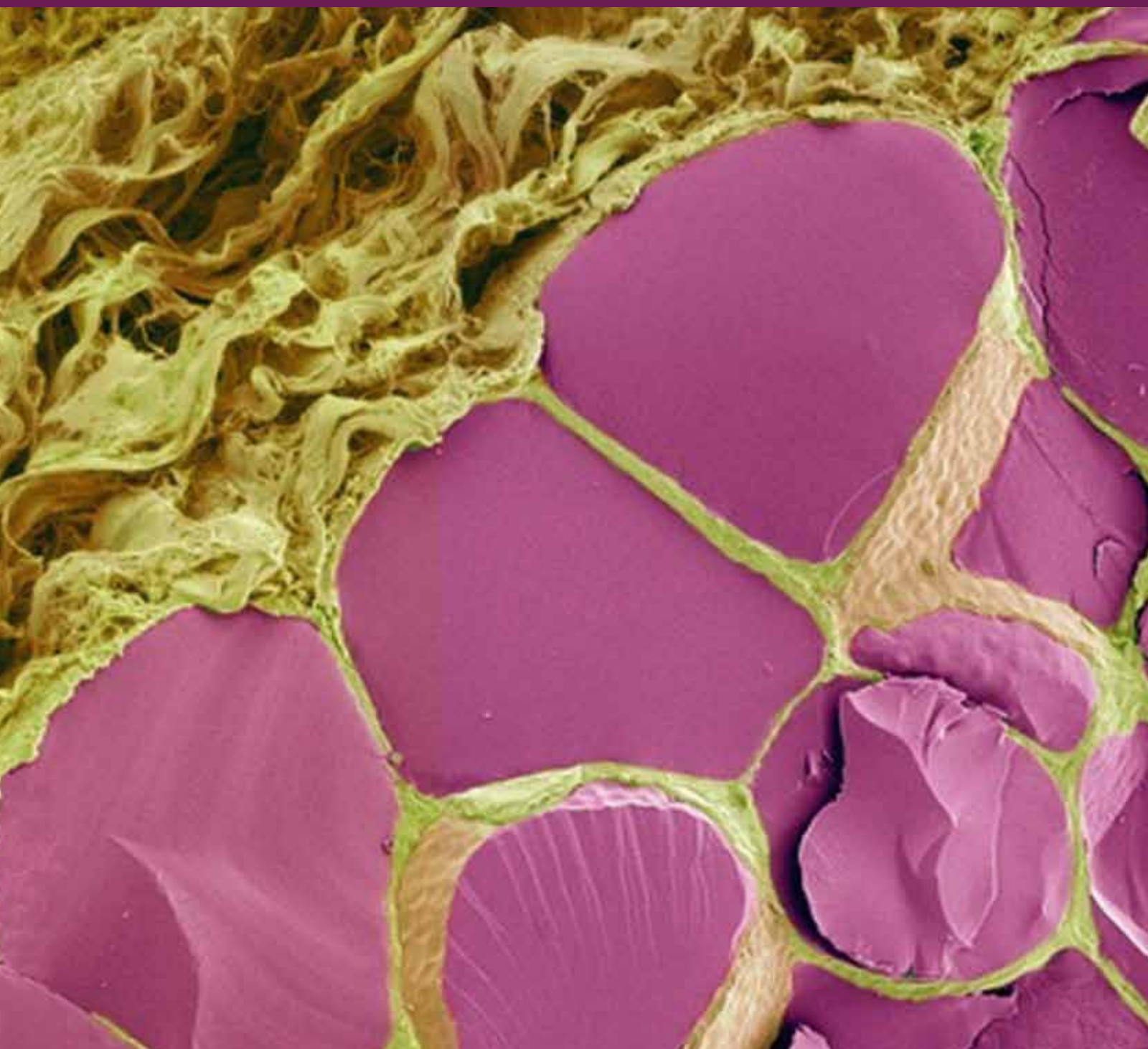


Endocrine Function in Aging

Guest Editors: Huan Cai, Alan S. Mcneilly, Louis M. Luttrell,
and Bronwen Martin





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International Journal of Endocrinology

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Editorial

Endocrine Function in Aging

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The endocrine system in higher mammals represents one of the most complex and fundamental systems that regulates nearly all of an organism's biological functions. This system is composed of multiple organs, tissues, hormones, and receptor modalities. Its ability to regulate critical functions such as reproduction, development, metabolism, stress responses, blood pressure, wakefulness, and digestion places it as one of the most important regulators of life-long physiology. Therefore, at any one point in time the physiological status of the majority of organs in the body is a function of the activity of the whole endocrine system. However, while appreciating the role of the endocrine system in such "frozen" points in time is important, the temporal variation in endocrine function across one's lifespan is of crucial interest to researchers investigating age-related disorders. The importance of gerontological research is becoming more and more evident, given the ever-increasing proportion of aged people in Western countries.

Aging is a natural process that involves a general decline in many physiological functions with time. Aging is generically associated with a reduced capacity to maintain homeostasis and effective repair mechanisms, resulting in loss of function, senescence, and eventually death. It is obvious that the functions of endocrine organs alter during the aging process, resulting in a higher prevalence of endocrine malfunction-related disorders in the elderly population. Enhanced knowledge and appreciation of endocrine functions in aging will likely lead to the development of successful pharmacological or lifestyle therapies to treat endocrine-related diseases in elderly patients. The discovery and development of novel endocrine-targeted remedies will hopefully

result in an improvement of quality of life and also overall lifespan. Thus, endocrine functions in the aging context are important fields of intense clinical and scientific interest and form the focus of this special issue.

The papers in this special issue are focused upon original research papers and review papers concerning several important molecular and tissue systems vital to the maintenance of the endocrine system in aging, that is, pancreatic function and type 2 diabetes mellitus (T2DM); testosterone deficiency and depression; metabolic and endocrine alterations in muscle dystrophies and sarcopenia; serum adipokines and osteocalcin in older patients with hip fracture; gerontological neuroendocrine axis organization and disruption; correlation of thyroid hormones and lipid profiles in elderly T2DM patients; minimally invasive approaches to parathyroid surgery in elderly patients; the proper interpretation of hormones and tumor marker measurements in the geriatric population.

As we have stated, aging is an important risk factor for metabolic disorders, including obesity, impaired glucose tolerance, and type 2 diabetes. Aging has long been associated in multiple animal species with the insulin/insulin-like growth factor-1 (IGF-1) signaling system. Z. Gong and R. H. Muzumdar summarize in their paper the current evidence on how aging affects pancreatic β -cell function, β -cell mass, insulin secretion, and insulin sensitivity. They also review the effects of aging on the relationship between insulin sensitivity and insulin secretion. Accelerated insulin resistance appears to be one of the strongest hallmarks of advanced physiological aging; therefore, a comprehensive understanding of all the defects that impair glucose homeostasis in the elderly

will likely lead to the development of novel treatments that may substantially improve life quality and lifespan.

Testosterone deficiency, or hypotestosteronemia, is a widely recognized hormonal alteration strongly associated with male aging. The review paper by M. Amore et al. comprehensively summarizes the current understanding of the correlation between depressive symptoms with a syndrome called partial androgen deficiency of the aging male (PADAM). This paper highlights the potential benefits of testosterone treatment upon mood and affective disorders. While supplementation with testosterone fails to show sound evidence of effectiveness in the treatment of depression, testosterone supplementation has proved to be effective, on some levels, for improving quality of life of aged patients with PADAM.

In addition to the gerontological effects upon steroid hormone activity, aging also significantly affects thyroid function. These age-related effects, acting through the thyroid hormone system, greatly impact both lipid profiles and somatic metabolic parameters. The study conducted by F. Strollo et al. investigates the correlation between free thyroxine (FT₄), free triiodothyronine (FT₃) levels and total cholesterol (TC), and low-density lipoprotein cholesterol (LDL-C) levels in euthyroid elderly T2DM patients. They found that TC and LDL-C correlate negatively with FT₄ and positively with FT₃. When divided according to treatment by oral hypoglycemic agents (OHA) and insulin (IT), they, however, reacted differently with respect to investigated associations.

Primary hyperparathyroidism (pHPT) is one of the most common endocrine diseases in the elderly and the chance of developing pHPT increases with age. Elderly patients with pHPT are often not referred for surgery because of their associated comorbidities that may increase surgical risk. The study by C. Dobrinja et al. demonstrates that minimally invasive approaches to parathyroid surgery seem to be safe and curative in elderly patients, with few associated risks because of the combination of modern preoperative imaging, advances in surgical technique, and advances in anesthesia care.

One of the most important factors related to the maintenance of health and independence in the elderly is endocrine-mediated control of the musculo skeletal system. An inability to maintain independence as well as increased morbidity due to elevated fall episodes are both likely to severely impact the cost of widespread healthcare in aging Western societies. Therefore, we have included several sections that contend with the effects of aging upon the endocrine regulation of musculo-skeletal tissues.

Common metabolic and endocrine alterations exist across a wide range of muscular dystrophies. The paper by O. del Rocío Cruz Guzmán et al. expertly reviews the current knowledge concerning the metabolic and endocrine alterations in diverse types of dystrophinopathies including childhood and adult dystrophies. K. Sakuma and A. Yamauchi also review the vital pieces of data concerning our current understating of the endocrine contribution to the age-related declines in muscle mass, muscle strength, and sarcopenia. These authors also investigated the current hormonal interventions designed to improve endocrine

defects related to sarcopenia. Myostatin inhibition seems to be an intriguing strategy for attenuating sarcopenia as well as muscular dystrophy. The authors discussed how therapeutic supplementation with growth hormone, IGF-I, or estrogen had a minor sarcopenia-inhibiting effect, and that testosterone supplementation in large doses had several side effects, even though it significantly improved muscle defects. Ghrelin mimetics could also potentially be beneficial and reverse the dysfunctional catabolic state associated with sarcopenia in the elderly population.

Low bone mass density, a classical age-related health issue and a known health concern for fair-skinned, thin, postmenopausal Caucasian women, is found to be common among individuals with developmental/intellectual disabilities. The review paper by J. Jasien et al. provides a comprehensive overview of bone health of adults with developmental/intellectual disabilities, their risk of fractures, and how this compares to the general aging population. The authors contend that gaining a greater understanding of how bone health affected in individuals with developmental/intellectual disabilities could lead to better customized treatments for these specific populations.

The paper by A. Fisher et al. reveals the interactions between serum adipokines and osteocalcin in older patients with hip fracture. The authors found that serum osteocalcin concentration was inversely associated with resistin and positively with leptin, leptin/resistin ratio, and adiponectin/resistin ratio after adjustment for multiple potential confounders. Osteocalcin was found to be an independent predictor of serum leptin, resistin, leptin/resistin, and adiponectin/resistin ratios, which suggests bidirectional interactions (crosstalk) between leptin, resistin, and osteocalcin as a part of a complex homeostatic system regulating bone and energy metabolism.

The accuracy of analytical measurements of different biochemical parameters is of vital importance for the proper diagnosis and treatment monitoring of elderly patients. The paper by K. Sztefko et al. discusses important points to be considered in the interpretation of hormone and tumor marker measurements in the geriatric population using immunochemical methods, including general lack of immunoassay standardization, presence of cross-reacting substances in patients' samples, limitation of free hormone measurements due to abnormal analyte binding protein concentrations, assay interferences due to a patient's autoantibodies, heterophilic antibodies, and proper interpretation of very low- and very-high-sample analyte levels.

While the endocrine system is classically associated with the regulation of autonomic hormonal functions, many lines of recent evidence have demonstrated that cognitive central nervous function in the elderly is significantly affected during the aging process by endocrine control of somatic metabolism. Hence, both normal and pathophysiological aging, as well as neurodegenerative disorders, are all influenced by this "neurometabolic" interface. This functional connection between these two important systems (neuronal and endocrine) is primarily mediated through hormonal communication between the brain and the metabolic organs. The review paper by S. Siddiqui et al. discusses the physical

structure and molecular components of this fundamental “neurometabolic” axis in aging. The authors then elaborate upon this by discussing how the connection of these two major functional domains is likely to be created by multifunctional “keystone” signaling factors, such as the epidermal growth factor receptor (EGFR). This paper draws together evidence to aid the appreciation of the truly multidimensional role of EGFR, at the systemic level, in neurometabolic processes and in the neurodegenerative trajectories seen in the aging process.

In another paper that discusses the functional interface between neuronal and endocrine systems during the aging process, A. M. Stranahan et al. investigate the effect of two well-characterized antiaging interventions (caloric restriction or exercise) upon hypothalamic function. The hypothalamus forms a vital bridge between higher neuronal activity and the status of the peripheral endocrine hormone system. Age-related changes in hypothalamic activity appear to be strongly connected to both endocrine and neuronal pathophysiological mechanisms. A. M. Stranahan et al. employ both caloric restriction and voluntary wheel running paradigms in diabetic and nondiabetic animals to investigate the contextual sensitivity of hypothalamic transcriptomic responses to these antiaging lifestyle strategies. The authors found that caloric restriction and physical exercise were associated with distinct hypothalamic transcriptional signatures that differed significantly between the host physiological contexts of the diabetic or nondiabetic mice.

In conclusion, our understanding of endocrine function in aging is making great strides. Several timely topics concerning endocrine function in aging were purposefully included in this special issue. However, it is clear that further efforts are needed to gain a greater appreciation of the mechanisms underlying endocrine alterations in aging, which will aid the development of more effective interventions for the treatment of endocrine defects during the aging process.

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Research Article

Free Triiodothyronine and Cholesterol Levels in Euthyroid Elderly T2DM Patients

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Thyroid function regulates lipid metabolism. Despite the fact that T2DM is more prevalent in the elderly, often associates with thyroid dysfunction and increases cardiovascular risk both *per se* and via high TC and LDL-C levels, the association of the latter with FT₃ and FT₄ levels has not yet been fully investigated in T2DM. While trying to fill this gap in 296 elderly outpatients with T2DM, we found that TC and LDL-C correlated negatively with FT₄ and positively with FT₃. When divided according to treatment by oral hypoglycaemic agents (OHA) and insulin (IT), they reacted differently with respect to investigated associations: in the OHA's TC and LDL-C correlated negatively with FT₄ and showed no association with FT₃, whereas, in the IT's TC and LDL-C correlated positively with FT₃ and negatively with FT₄. When controlled for possible confounding factors, these associations did not change in the IT's but were missing in the OHA's. Recent literature reports upon complex hypothalamic and peripheral interactions between T2DM and thyroid, and suggests T₃ to enhance cholesterol synthesis and to have a role in insulin resistance states. Further investigations are needed to understand the intimate mechanisms of lipid metabolism in T2DM with respect to thyroid function.

1. Introduction

Thyroid function affects both lipid profiles and metabolic parameters [1]. Thyroxine (T₄) and triiodothyronine (T₃), the two main thyroid hormones, are secreted after thyroglobulin macropinocytosis and hydrolysis under the stimulation of pituitary thyrotropin (TSH) and are present in the blood mainly in noncovalent interactions with thyroxine-binding globulin, prealbumin, and albumin. Only 0.4% T₃ and 0.04% T₄ dynamically escape binding and, since free to interact directly with peripheral organs and tissues, are called free T₃ (FT₃) and free T₄ (FT₄) and are the only fractions believed to be metabolically active [2].

FT₄ levels are about 100 times higher than those of FT₃, which is considered to be the most active form of thyroid hormone and into which FT₄ is converted within

the peripheral tissue [3]. Inside a variety of cells, both interact with different affinity with $\alpha 1$, $\alpha 2$, $\beta 1$, and $\beta 2$ nuclear receptors in regulating the expression of target genes through the so-called thyroid response elements (TRE's), thus exerting the typical physiological functions which characterize them. These are mostly represented by enhanced calorogenesis, increased stroke volume and heart rate, enhanced sensitivity to catecholamines, bone turnover, gluconeogenesis, glycogenolysis, and lipolysis. The latter is mainly due to enhanced lipoprotein-lipase activity and increased hepatic low-density lipoprotein (LDL) receptor concentrations [4]. Besides genomic effects, widespread rapid onset nongenomic effects of FT₃ and FT₄ have been reported involving membrane-signalling pathways [5], the real extent of which has not been well defined yet [6].

When dealing with lipid metabolism, circulating T_4 concentrations have always been found to be inversely associated with total (TC), high-density lipoprotein (HDL-C), and LDL cholesterol (LDL-C) levels [7]. In fact, high TC levels in hypothyroidism are caused by a reduction in LDL receptors [8] and L- T_4 administration has a hypolipidemic effect in hypothyroidism, which is characterized by high thyrotropin (TSH) concentrations, meant at compensating for low FT_4 levels. Thus, there is a greater benefit in patients displaying higher pretreatment TC or LDL-C and TSH despite the difficulty to identify any well-defined cut-off threshold for the association of the latter with lipids [9]. As a matter of fact, 4.3% hypercholesterolemic patients have been reported to be hypothyroid [10].

A contributing factor to high cholesterol levels in hypothyroidism is represented also by low FT_3 . Under normal conditions, the latter has the control over sterol regulatory element-binding protein-1 (SREBP-1), a crucial step for the expression of the LDL receptor, rather than over SREBP-2 [11]. However, it also acts directly by inducing at least two LDL-receptor TRE's in the liver [12]. Finally, increased flow of bile acids as a result of FT_3 and FT_4 is known to reduce cholesterol levels by depletion of its hepatic pool; however, such effect is counterbalanced by enhanced synthesis and uptake in the liver, eventually increasing TC and LDL-C under unfavourable circumstances [13].

Based on the considerations mentioned above, the relationship between thyroid function and the metabolic syndrome (MetS) has become a subject of interest for many research groups during the last few years [14], leading to the conclusion that even low-normal T_4 levels may contribute to increased cardiovascular risk associated with lipid abnormalities in people with the MetS [15]. Some of these studies reported that T_3 behaved in a different way, being positively associated with body mass index (BMI) and waist girth [16–18]. A higher compensatory conversion of T_4 to T_3 to increase thermogenesis in obesity was the hypothesized mechanism [19, 20].

Diabetes mellitus (DM), in particular type 2 (T2DM), which is mostly associated with lipid abnormalities and displays an increasing prevalence with age [21], is also known to dramatically increase cardiovascular risk (CVR) at any age [22]. Age does not seem to affect FT_3 and FT_4 levels in the human [23], whereas people with DM have been reported to suffer from thyroid dysfunction twice as much as the nondiabetic population [24]. Nevertheless, despite the fact that TC and LDL-C are major CVR factors identified as crucial targets in all current diabetes treatment guidelines [25], no extensive investigation can be found in the literature concerning the relation between thyroid and lipids in T2DM. This link is missing in the clinical management of people with DM now that carbohydrate response element-binding protein (ChREBP)—a newly identified lipogenic glucose-sensing transcription factor controlling hepatic lipogenesis—is known to be positively controlled by T_3 in mammals [26] through its binding to thyroid receptor β -1. In fact, T_3 has been shown to modulate hepatic lipogenesis through reciprocal regulation of SREBP-1c and ChREBP gene expression [27]. Moreover, T_3 is endowed with

a lipogenic effect via ChREBP enhancement in white adipose tissue, where both α and β thyroid receptors are expressed but only the β isoform is active with regard to that effect [28]. Thus, glucose, lipids, and thyroid hormones seem to interact according to a more complex mathematical function than as previously expected.

Taking into account the considerations mentioned above, with the present study, we evaluated the relations among FT_3 , FT_4 , TC, and LDL-C in elderly euthyroid people with T2DM.

2. Materials and Methods

We retrospectively evaluated clinical records from 350 outpatients, ages 70 years and older, referred to our clinic for T2DM during the last three years. All of them had thyroid function routinely evaluated in terms of FT_3 , FT_4 , and TSH concentrations. The study protocol was approved by the ethical committee. Exclusion criteria included smoking and any complications worse than background retinopathy, microalbuminuria, low-grade neuropathy, and nonobstructive arteriopathy. We also did not accept for this study patients with overt heart, liver or kidney failure, hypo/hyperthyroidism, or those on any medications possibly interfering with thyroid function. Thus, 296 people were qualified for study (245 women, 51 men), all taking statins since the time of diagnosis (4.5 ± 2.7 years) in order to prevent cardiovascular complications according to Italian Diabetes Guidelines [29]. They were either under oral hypoglycaemic agents (OHA subgroup, $n = 196$), invariably consisting of sulphonylureas and metformin, or under insulin treatment (IT subgroup, $n = 100$, of which 63 on 4 basal-bolus injections, the others with 2 or 3 injections as needed). Both subgroups followed a thorough self-monitoring blood glucose supervision associated with a strong empowering strategy [30].

Blood was drawn in our laboratory in the morning, after a 12-hour overnight fast. Chemistry was measured by Kodak Blood Multiple Analyzer and thyroid hormones by Immulite 2000 Immunoassay System.

Data evaluation was based upon SPSS 13.0 for descriptive (mean \pm SD) and correlation analysis. Correlation analysis was performed first on all cases and then completed by partial correlation analysis applied to each subgroup (namely, OHA and IT), controlling for possible confounding factors. The least statistical significance of the differences among the means and of the associations was set at $P < 0.05$.

For clarification concerning the abbreviations used within the text, please refer to the list at the end of the paper.

3. Results

The means and the standard deviations (S.D.) of all recorded clinical parameters are presented in Table 1.

When analysing all the cases, we observed a positive correlation of FT_3 with both TC ($r = 0.144$, $P < 0.05$) and LDL-C ($r = 0.161$, $P < 0.02$). Conversely, FT_4 correlated negatively with TC and LDL-C ($r = -0.131$, $P < 0.05$ and $r = -0.134$, $P < 0.05$, resp.). The disease duration

TABLE 1: Clinical parameters (mean \pm SD) in the 296 patients under evaluation.

	Mean	SD
Age (years)	76.4	5.2
BMI (Kg/m ²)	31.5	7.4
Dis-Dur (years)	4.47	2.69
HbA1c (%)	7.95	2.08
TC (mg/dL)	188.5	40.4
HDL-C (mg/dL)	47.6	14.3
LDL-C (mg/dL)	111.0	35.5
TG (mg/dL)	148.6	81.4
SBP (mmHg)	136.1	16.5
DBP (mmHg)	74.8	11.4
FT ₃ (pg/mL)	2.76	0.77
FT ₄ (pg/mL)	12.5	5.9
TSH (mU/L)	1.44	1.35

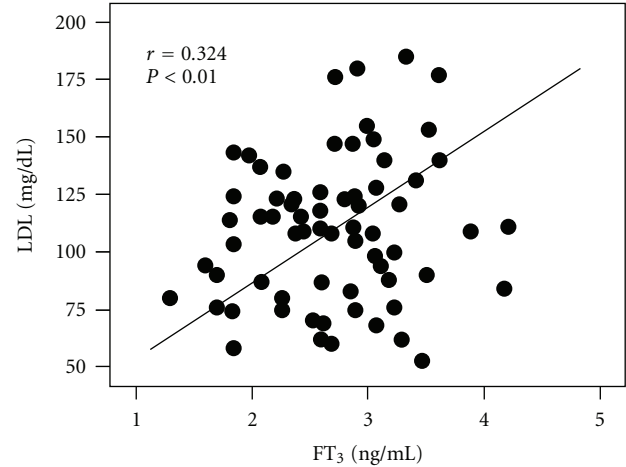
TABLE 2: Clinical parameters recorded in the IT and OHA patients: none of them differs significantly between subgroups.

	IT subgroup		OHA subgroup	
	Mean	SD	Mean	SD
Age (years)	76.2	4.9	76.5	5.2
BMI (Kg/m ²)	30.9	6.4	31.9	7.9
Dis-Dur (years)	5.01	2.80	4.13	2.47
HbA1c (%)	8.0	2.5	7.9	2.1
TC (mg/dL)	184.6	41.6	191.0	40.3
HDL-C (mg/dL)	46.1	13.2	48.6	14.8
LDL-C (mg/dL)	107.5	35.1	113.2	36.2
TG (mg/dL)	150.2	67.5	144.1	74.4
SBP (mmHg)	137.1	16.6	135.7	16.6
DBP (mmHg)	73.8	12.4	75.4	11.1
FT ₃ (pg/mL)	2.73	0.94	2.80	0.65
FT ₄ (pg/mL)	11.9	2.1	12.8	7.1
TSH (mU/L)	1.45	1.40	1.37	1.29

and HbA1c were found to correlate negatively with LDL-C ($r = -0.108$, $P < 0.05$ and $r = -0.144$, $P < 0.02$, resp.). To avoid confounding effects, we introduced them together with other potentially interfering factors that are possibly related to thyroid function (age, BMI, and blood pressure) into partial correlation analysis of total and LDL-C with FT₃ and FT₄. As a result, no changes in correlation coefficient signs or significances were observed.

At this point, when we proceeded to further analyze the variance of observed parameters between OHA and IT subgroups, we found that the two were statistically homogenous with each other, as shown in Table 2.

Though, in terms of correlation analysis, the two subgroups behaved differently in terms of thyroid hormones. In fact, in the OHA, total and LDL cholesterol showed no correlation with FT₃ ($r = 0.037$ and $r = 0.098$, resp., nonsignificant) but correlated negatively with FT₄ ($r =$

FIGURE 1: Association between FT₃ and LDL cholesterol in IT-treated T2DM subjects controlling for age, BMI disease duration, blood pressure, and HbA1c.

-0.144 , $P < 0.05$ and $r = -0.145$, $P < 0.05$). Conversely, in the IT, total, and LDL cholesterol correlated positively with FT₃ ($r = 0.291$, $P < 0.01$ and $r = 0.275$, $P < 0.02$, resp.) while maintaining the same negative correlation with FT₄, as observed in the OHA ($r = -0.239$, $P < 0.05$ and $r = -0.186$, $P < 0.05$). Moreover, the IT revealed a previously “hidden” negative correlation between HDL cholesterol and FT₄ ($r = -0.302$, $P < 0.01$).

We then repeated the same correlation analysis controlling lipid to thyroid relationship for age, BMI, HbA1c, blood pressure, and disease duration within each subgroup. As clearly shown in Figure 1, we could therefore confirm the positive correlation of FT₃ with LDL cholesterol. Moreover, as summarized in Table 3, all the correlations previously described before were confirmed, including the one linking FT₄ to HDL cholesterol.

4. Discussion

Before discussing our results, we will try to summarize them. TC and LDL-C displayed opposite correlations with FT₃ (positive) and FT₄ (negative) in elderly euthyroid subjects with T2DM. While a negative correlation would have been easy to accept and understand for thyroid hormones, a positive one was totally unexpected according to current concepts. When repeating the analysis in the two different subgroups, controlling them for confounding factors, we confirmed the above findings in the IT subgroup but not in the OHA subgroup.

However, regarding the clinical implications of our results, a crucial point is that elderly people with T2DM carry the burden of a high CVR and have to be treated as carefully as their younger counterpart since morbidity/mortality increases together with their LDL-C levels. With this in mind, diabetologists often concentrate on glucose, lipids, and blood pressure and generally do not take into account thyroid hormones in the absence of typical symptoms of

TABLE 3: Correlation coefficients of cholesterol and its LDL and HDL fractions to FT₃ and FT₄ in T2DM elderly patients controlling for age, BMI, disease duration, and HbA1c.

Control variables	Thyroid hormone	Group	Statistics	Total cholesterol	LDL cholesterol	HDL cholesterol
Age, BMI, disease duration, blood pressure, and HbA1c	FT ₃	IT	<i>r</i>	0.346	0.324	0.147
			<i>p</i>	0.006	0.009	n.s.
		OHA	<i>r</i>	-0.014	0.043	-0.038
			<i>p</i>	n.s.	n.s.	n.s.
	FT ₄	IT	<i>r</i>	-0.295	-0.241	-0.310
			<i>p</i>	0.019	0.050	0.013
		OHA	<i>r</i>	-0.149	-0.142	-0.119
			<i>p</i>	n.s.	n.s.	n.s.

IT: insulin treated; OHA: treated by oral hypoglycaemic agents.

thyroid dysfunction [31]. On the other hand, subclinical hypo- and hyperthyroidism are not rare at all. As geriatric endocrinologists, we prefer to assay thyroid hormones in diabetic elderly patients in order to rule out any subtle thyroid malfunction during the course of overall evaluation.

Such a habit allowed us to collect data from a number of euthyroid elderly people with T2DM and to perform a correlation analysis between cholesterol and thyroid hormones. With regard to FT₄, the analysis confirmed that thyroid function negatively correlates with TC and LDL-C, a trend commonly found in the general population. However, quite unexpectedly FT₃ behaved the opposite way, being positively associated with TC and LDL-C. We then analyzed the association between thyroid hormones and all possible confounding factors. We found that age correlated positively and HbA1c and negatively to TC and LDL-C. Therefore, we introduced these two factors and other potentially interfering parameters including BMI, blood pressure, and disease duration as confounding factors, confirming their associations by performing partial correlation analysis.

After we divided our subjects into OHA and IT subgroups, the analysis of variance showed that they were fully homogeneous in terms of recorded clinical parameters. The only difference was in their treatment, namely, metformin/sulphonylureas versus insulin. This made us more confident in trying to reveal eventual differences occurring in relations between lipid profile and thyroid hormones. In fact, this would allow us to check whether different associations within subgroups are possibly linked to different treatment regimens.

In the OHA group, TC and LDL-C displayed no correlation with FT₃ but correlated negatively with FT₄ ($P < 0.05$), whereas in the IT group, TC and LDL-C correlated positively with FT₃ ($P < 0.02$) and negatively with FT₄ ($P < 0.05$). Once again we performed partial correlation analysis in the IT subgroup and found that the positive association of lipids with FT₃ became even stronger while that with FT₄ remained the same (see Table 3); however, both vanished in the OHA subgroup.

Such findings might seem contradictory, but, in fact, they fit well with the role played by FT₃ on SREBP-1 and SREBP-2 control, and consequently on the expression of LDL receptor, with its ability to indirectly enhance cholesterol

synthesis and uptake by the liver. Moreover, in some previous studies an unexpected positive association between FT₃ and lipids was mentioned without indepth explanation, and therefore remained underestimated. For instance, De Pergola et al. [32] found that FT₃ correlated negatively with HDL-C levels ($P < 0.001$), and, in multiple correlation analysis, maintained an independent positive association with age ($P < 0.001$), waist girth ($P < 0.05$) and insulin levels ($P < 0.001$), a proxy for insulin resistance. It is worthwhile noting that in the study of De Pergola et al., FT₃ was also associated with smoking habits. Our study took into account only nonsmokers, thus ruling out *a priori* a possible strong confounding factor. Others confirmed the previously mentioned association between FT₃ and MetS components [15] suggesting insulin-resistance to be the link between the thyroid and lipids. Therefore, T₃ may act as a strong independent metabolic signal in euthyroid insulin-resistant T2DM patients [33, 34].

Interestingly enough, according to recent reports, T₃ added to diets containing peanut oil increased serum lipids in rats, sometimes even up to 20-fold [35], whereas T₃ was found to increase cholesterol biosynthesis in the liver through the activation of *de novo* protein synthesis [36, 37]. Furthermore, T₃, insulin, and their combination markedly stimulate cholesterol synthesis in cultured human skin fibroblasts [38], and in recent studies FT₃ has been even shown to exert a beta-cell protective effect [39, 40]. All above considerations might explain the association we found between FT₃ and TC and LDL-C in our IT patients and not in our OHA patients. In fact, it seems as if in the presence of insulin resistance T₃ may not be able to act as fully as in the case of an unopposed insulin signal, such as when metabolically effective exogenous insulin levels are attained. Still another possible explanation for our findings result from an eventual compensatory increase in FT₃. In fact, based upon widely accepted lipid metabolism regulation mechanisms by thyroid hormones, it might be hypothesized that in our patients cholesterol synthesis might have been enhanced by exogenous insulin and by peripheral T₃ conversion from T₄ [41, 42].

Another apparently anomalous finding, regarding the negative correlation of HDL-C with FT₄ levels ($P < 0.01$) but not with FT₃, deserves more discussion. Since early

studies in the field, HDL-C was found to decrease both in hypo- and in hyperthyroidism, thus indicating that such association followed a U-shaped curve and could, therefore, only be analyzed within the physiological range of FT₄ concentrations [43, 44]. In fact, slightly different behaviour between the two hormones has been reported in the literature, including a stronger enhancing effect of HDL-C on T₄ target cell penetration in comparison to T₃ target cell penetration [45]. A pro-HDL and anti-LDL effect of T₃ was also described [46]. These observations have been often overlooked, despite their potential pharmacologic utilization in the metabolic syndrome.

We are aware of the intrinsic limits of the retrospective cross-sectional character of our study, which refers to subjects over 70 years of age with T2DM and, therefore, allows no definite conclusions with regard to younger people or in terms of cause-effect relationships. Nevertheless, due to the different association patterns found in IT patients as compared to OHA patients, we feel it is worthwhile to further investigate the topic.

Specifically, reported data prompts some reappraisal regarding the relationship among some hidden aspects of cholesterol, insulin, and thyroid hormone metabolism, eventually fostering new controlled studies concerning the role of T₃ in T2DM and the possible pharmacological interferences that different drugs may have on it.

Based on our data, it seems more prudent to treat elderly hypothyroid patients with T₄, without any T₃ integration if they are on insulin. This might prevent the risk of letting their LDL-C increase and thus of increasing statin dosage. We still do not fully understand the meaning of the association we found between FT₃ and cholesterol in IT people with T2DM. In other terms, the positive association might be the expression of a compensatory hormone response to spontaneous LDL-C increase, as already hypothesized for patients with the MetS [15, 33, 34] and in line with the complex DM-thyroid interactions occurring via hypothalamic glucose sensing mechanisms [47] (as recently reviewed by Duntas et al. [48]).

In conclusion, in order to clarify the pathophysiological mechanisms underlying the observed results further experimental and clinical follow-up studies are needed. Should the observed associations be confirmed by future investigations, in older T2DM patients it might be useful to include FT₃ once again in thyroid test panels which today are mostly limited to screening TSH and confirmation FT₄.

Abbreviations

BMI:	Body mass index
ChREBP:	Carbohydrate response element-binding protein
DBP:	Diastolic blood pressure
Dis-Dur:	Disease duration
DM:	Diabetes mellitus
FT ₃ :	Free triiodothyronine
FT ₄ :	Free thyroxine
HbA1c:	Glycated haemoglobin
HDL:	High density lipoprotein

HDL-C:	HDL cholesterol
IT:	Insulin treated
LDL:	Low-density lipoprotein
LDL-C:	LDL cholesterol
OHA:	Oral hypoglycaemic agents
SBP:	Systolic blood pressure
SREBP:	Sterol regulatory element-binding protein
T2DM:	Type 2 diabetes mellitus
T ₃ :	Triiodothyronine
T ₄ :	Thyroxine
TC:	Total cholesterol
TG:	Triglycerides
TRE:	Thyroid response element
TSH:	Thyroid stimulating hormone.

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Review Article

Aging and Bone Health in Individuals with Developmental Disabilities

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Low bone mass density (BMD), a classical age-related health issue and a known health concern for fair skinned, thin, postmenopausal Caucasian women, is found to be common among individuals with developmental/intellectual disabilities (D/IDs). It is the consensus that BMD is decreased in both men and women with D/ID. Maintaining good bone health is important for this population as fractures could potentially go undetected in nonverbal individuals, leading to increased morbidity and a further loss of independence. This paper provides a comprehensive overview of bone health of adults with D/ID, their risk of fractures, and how this compares to the general aging population. We will specifically focus on the bone health of two common developmental disabilities, Down syndrome (DS) and cerebral palsy (CP), and will discuss BMD and fracture rates in these complex populations. Gaining a greater understanding of how bone health is affected in individuals with D/ID could lead to better customized treatments for these specific populations.

1. Introduction

Developmental disability (DD) is a group of severe chronic conditions that are attributable to an impairment in physical, cognitive, speech, language, psychological, or self-care areas that are manifested during the developmental period (younger than 22 years of age) [1]. Intellectual disability (ID) is a disability characterized by significant limitations both in intellectual functioning and in adaptive behavior, which covers many everyday social and practical skills. This disability originates before the age of 18 [2]. ID has been classified through performance on IQ tests as mild (IQ 69–55), moderate (IQ 54–40), severe (IQ 39–25) or profound (IQ <25). These cutoffs are typically based on tests with a mean score of 100 and a standard deviation of 15 and do not reflect the standard error of measurement, which is approximately

5 points. If an individual is intellectually disabled, they could also be identified as having a developmental disability. However, one can possess a developmental disability, such as a motor or language disability, and not be intellectually disabled. Down syndrome (DS) and cerebral palsy (CP) are two examples of DD. DS individuals are intellectually disabled, and approximately fifty percent of individuals with CP have an intellectual disability.

The life expectancy of the general healthy population has increased significantly over the past decades. Although many studies have investigated the aging process in the general population, relatively little attention has been paid to aging in people with developmental disability/intellectual disability (D/ID). Rigorous and robust studies that investigate the aging process in individuals with D/ID are currently lacking. The exact reasons for this disparity in aging research are

unclear, but a change in society's approach to the care of individuals with D/ID has likely been a major contributing factor. Prior to the 1970s, many individuals with D/ID did not live long enough to complete rigorous aging studies and individuals with D/ID were predominantly deinstitutionalized in the United States. In 1983, Carter and Jancar examined trends in the causes of death and mortality rates in patients with "mental handicaps" residing in an institution in the United Kingdom between 1930 and 1980. From 1931 to 1935, the average life expectancy for males with D/ID was 14.9 years and 22.0 years for females with D/ID. Poor sanitary conditions and nutrition, lack of adequate medical care, and crowding in the institutions during that period have been attributed to this disparity in aging [3]. Tuberculosis was a major cause of death until the 1950s, and the life expectancy increased slowly over the years from 1971 to 1975, until it was approximately 49.8 years for males and 54.1 years for females with D/ID [3]. This trend of increasing longevity has also been seen in the DS population; people with DS experienced a doubling in life expectancy over a 14-year span [4]. In 1983, the average lifespan for an individual with DS was 25 years, and by 1997 it had increased to 49 years [5]. This doubling of life expectancy has been attributed to numerous factors, such as improved medical interventions during childhood (e.g., cardiac surgery), improved living environments, diets, and illness interventions such as diagnosis and treatment of hypothyroidism [6–8]. Currently, the life expectancy for many individuals with D/ID is similar to that of the general healthy population, except for adults with certain genetic/metabolic conditions and with a more severe intellectual disability. For mild to moderate developmental disabilities, life expectancy is approximately 70 years, although for DS and severe developmental disabilities it is approximately 50 years [4, 9, 10].

The reason for the persistent shorter lifespan for the DS population, compared to many of the other developmental disabilities, is thought to be due to an accelerated aging process, which is manifested by increased rates of cataracts, hearing loss, osteopenia, hypothyroidism, and a genetically elevated risk for developing Alzheimer's disease [11]. The population of adults with an intellectual and developmental disability aged 60 and older is projected to double from 641,860 in 2000 to 1.2 million by 2030. This could be attributed to an increasing life expectancy and aging of the "Baby Boomer" generation [12]. Due to this increased longevity, individuals with D/ID are confronted by many of the same chronic illnesses that affect the general aging population, but their onset may be earlier and the effects diverge in severity. Effective studies to determine this however are currently lacking, and there is a dearth of informative literature on this topic. The aim of this paper is to provide an overview of the present literature on some of the aging aspects of intellectual and developmental disabilities. We will specifically focus on a key aspect of the aging process, bone health and bone mass density (BMD), in two important and common developmental disabilities: DS and CP. First, we will provide a brief overview of bone health and fractures in the normal aging population; next, we will provide an overview of studies on bone health in adults with D/ID, including

BMD, risk factors for low BMD, fracture rates, and a limited discussion of treatment studies. Lastly, bone health in the DS and CP populations will be discussed.

2. Bone Health in the Normal Aging Population

Osteoporosis is one of the most common conditions associated with aging in the general population [13]. Osteoporosis is a disease of bones that leads to an increased risk of fracture. In osteoporosis the BMD is reduced, bone microarchitecture deteriorates, and significant protein expression alterations in bone are apparent. Osteoporosis is defined by the World Health Organization (WHO) as a bone mineral density that is 2.5 standard deviations or more below the mean peak bone mass (average of young, healthy adults) as measured by dual-emission X-ray absorptiometry (DXA). Using DXA, two measures of bone health are typically obtained: a *T* score and a *z* score. According to the WHO definition, a *T* score is the comparison of a person's bone density with that of a healthy 30-year old of the same sex. Osteoporosis is defined by a *T* score of -4.0 to 2.5 , osteopenia is -2.5 to -1.0 , normal bone mass is -1.0 to 1.0 , and high normal bone mass is 1.0 to 4.0 . The *z* score is a comparison of a person's bone density with that of an average person of the same age and sex. Bone is being continuously turned over in distinct areas of the skeleton due to bone-forming osteoblasts and bone-resorbing osteoclasts [14, 15]. In healthy young adults, bone resorption and formation are tightly linked, thereby maintaining a steady state of bone [15]. However, during the aging process, significant bone loss occurs due to the tipping of this finely tuned equilibrium towards enhanced resorption, coupled to decreased bone formation [15, 16]. This net loss in bone mass during the aging process can ultimately lead to osteoporosis [15]. A multitude of factors are known to play a role in maintaining adequate bone health, including nutrition, lifestyle choices, genetics, and hormonal status [16].

A recent observational cross-sectional study analyzed risk factors in aging subjects with a recent clinical fracture [17]. Over the course of one year, men and women over fifty years of age who presented to a medical facility with a clinical fracture were invited to participate in a bone- and fall-related risk factor assessment and receive a bone density measurement. The bone-related risk factors for fracture assessment included a previous fracture after the age of fifty, a mother with a fracture history, a body weight of <60 kg, severe immobility, and the therapeutic use of glucocorticoids. The fall-related risk factors for fractures included more than one fall in the past year, the use of psychoactive drugs, a low level of activities of daily living before the current fracture, articular symptoms, impaired vision, urinary incontinence, and Parkinson's disease. This fracture risk factor assessment was based on the Dutch guidelines for the prevention of osteoporosis and falls [17]. Patients were excluded if they were receiving treatment for osteoporosis or had a pathologic fracture. This study comprised 406 women with a mean age of 68 years and 162 men with a mean age of 65 years. It was found that the prevalence of fall-related risk

factors (75%, $n = 425$) and the prevalence of bone-related risk factors (53%, $n = 299$) at the time of fracture were higher than the prevalence of osteoporosis (35%, $n = 201$). Fall- and bone-related fracture risk factors were present and independent of fracture location, age, or gender. Fifty percent of the patients had an overlap between bone and fall-related risk factors. After adjusting for age, weight, and height, women with a fracture were found to more frequently have a diagnosis of osteoporosis and have a more frequent history of falls than did postmenopausal women without a fracture history. This study implies that in order to predict fractures in an aging population, knowing bone-related and fall-related risk factors could be just as important as actual BMD measurements [18]. Additionally, immobility was also found to be a significant risk factor for recurrent fractures in the normal elderly population [19]. Fractures in the normal elderly general population not only lead to pain and immobility but also mortality and institutionalization. In a study seeking to identify determinants of mortality and institutionalization after hip fractures and also hip fractures in patients at high risk of death or institutionalization after hip fracture, cognitive impairment was found to increase the chances of mortality and institutionalization [20]. In this study, male gender was found to also increase mortality risk fourfold. Patients with lower postfracture physical function had at least five times the risk of institutionalization, compared to patients with high postfracture physical function [20].

Osteoporosis not only affects women in the normal aging population but also men, and it can have detrimental effects. Estrogen deficiency appears to be a major factor in the pathogenesis of osteoporosis in both genders [13]. However, mortality after a hip fracture, one of the major complications of osteoporosis, is more common in men than in women. Some of the risk factors for low BMD in males that have been assessed include calcium intake, exercise, alcohol consumption, and smoking [21]. Age-related bone loss and osteoporosis generally put the elderly population at an increased risk for fractures and morbidity. However, our understanding of age-related bone loss in the normal healthy population has increased greatly in recent years and has led to better diagnoses and treatments. This is not the case for bone loss in other populations, such as those with developmental disability, and more studies are needed to investigate bone loss in these populations.

3. Bone Health in the Adult Developmental/Intellectual Disability Population

3.1. Low Bone Mass Density Prevalence. It is a consensus that adults with D/ID have low BMD. Most of the available studies do not provide T or z scores for various age groups, but rather a mean for the entire study population. Other studies meanwhile do not provide T or z scores at all. In a BMD study of 94 individuals with mild to severe ID (53 females and 41 males, 12 with DS but no gender specified) with a mean age of 35 living in the community, it was found that females possessed a mean z score of lumbar sacral spine of

-0.6 and males a mean z score of -0.4 [22]. Since research of BMD in individuals with D/ID had been restricted to small population sizes, Zylstra et al. conducted a cross-sectional study to investigate the prevalence of osteoporosis in a larger population living in the community [23]. This study comprised 298 individuals (167 males and 131 females) with mild to profound ID aged 6–90 years. The rate of osteoporosis of the femur bone was 17.1% and the rate of osteopenia was 51.0%. Additionally, the mean T score was found to be -1.71 for all the ages and both genders. Osteoporosis rates for individuals aged 45 or younger were significantly less than those for individuals aged 46 and older (36.6% versus 48.4%). Although the population size was small in the >65-year-old subgroup, the investigators found that the females in this age group had fewer low BMD scores that met the criteria for osteoporosis than the men. Hence, 3 out of 12 (or 25%) females and 3 out of the 7 males (or 43 %) aged 66 and older met criteria for osteoporosis, which was similar to results reported previously [24]. It was found in the Zylstra [23] study that 19.2% of males with ID had osteoporosis, compared to 14.5% of females with ID. Another study uniquely compared how many individuals carried the diagnosis of osteoporosis prior to a DXA measurement [25]. In this study 107 adults with D/ID aged 40–60 years, living in the community, were investigated. Only 1% of the entire sample had a preexisting diagnosis of osteoporosis and only 4% were taking calcium supplements, while 34% of the subjects were found to be osteopenic and one-fifth of the group (21%) was found to be osteoporotic. A research cohort of 108 institutionalized men (mean age of 52) with intellectual disability, CP, or autism had a mean T score of -1.96 and an average z score of -1.30 . 34% of this study group had a z score of ≤ -2 below those of age-matched controls. No steady decline of mean z scores by age groups was apparent, but rather a variability of scores across ages was observed: 20–29 years: -0.68 , 30–39 years: -1.92 , 40–49 years: -1.37 , 50–59 years: -1.40 , 60–69 years: -1.05 , 70–79 years: -0.95 , and 80+ years: -1.25 [26]. An additional study including both institutionalized men and women with ID found that 28 out of 50 males (mean age of 54) and 32 of 58 females (mean age of 53) had broadband ultrasound attenuation results 2 SD units below the expected mean value for the patients' age [27]. Seven of these individuals were given a DXA and 4 were found to have lumbar spine or femoral neck T scores of more than 2.5 SD units below the mean for the same gender, with a trend of lower T scores for the males [27]. A larger sample of 562 adults with D/ID living in a long-term care facility from ages 30 to 65+ (mean age 45) had a mean T score of -0.8 and mean z score of -0.85 for all participants. Out of the 191 males, 10 were older than 65 and 4 (or 40%) had osteoporosis and 1 (or 10%) had osteopenia. Out of 96 females, 5 were older than 65 and 3 (or 60%) had osteoporosis and 1 (or 20%) had osteopenia [28]. In a later study (132 men and 79 women), it was found that more than three quarters (77%) of the study population had a low BMD [28]. In this report the mean age for women was 72.1 years, 73.8 for men aged over 60, and 42.7 years for men under age 60. Of the participants, 35.5% had mild to moderate ID and 64.4% had severe to

profound ID. No actual *T* scores were provided, but the authors reported that 62% of participants of 60 years and older had *T* scores of < -2.5 and one-third had *T* scores of -1 to -2.5 . Stratified by gender, 67% of women aged 60+ were osteoporotic, compared to 48.5% of men in the same age group. It was found that 26% of men under 60 years were osteoporotic and 36% were osteopenic. Among residents of 60 years and older, women were found to be three times more likely than men to have low BMD [28]. These various cross-sectional studies indicate that adults in the community setting and institutions with D/ID have low BMD. The natural history and mechanisms underlying this condition for this population, however, remain unclear. There is an evident trend of lower BMD in adult males than females with D/ID, although this is not statistically significant and has not been demonstrated in all of the clinical studies.

3.2. Low Bone Mass Density Risk Factors. Several risk factors for bone loss and low BMD that have been characterized in the general aging population have also been assessed in people with D/ID. Similar to the Dutch guidelines for the prevention of osteoporosis and falls [17], age [22–24, 28], body weight [22], and immobility status [23, 24, 28] were assessed, and all three of these factors were found to be associated with low BMD. Age greater than 60 [29] and 65 years [23, 24] was associated with lower BMD in both genders, although it has to be pointed out that in the Jaffe and Timell study, only women 60 years and older were included. Similar to the general population, Caucasians with D/ID were found to be at higher risk for low BMD [23, 24]. Additionally, circulating hormone levels were assessed in one study and hypogonadism was found to be associated with low BMD in females [22]. Unique risk factors assessed in the adults with D/ID include phosphate in women [22], degree of intellectual disability [23, 24, 28], antiepileptic drug use [28], DS status [22, 25], and hypothyroidism [25]. All of these unique factors were associated with low BMD in adults with D/ID, except hypothyroidism; however, hypothyroidism was only assessed in one study [25]. Interestingly, the level of ID that was found to be most strongly associated with low BMD was profound ID [23, 24, 28].

3.3. Bone Mass Density Measurements. The DXA scan is the standard measurement technique of BMD of the hip and lumbar spine in the general population, but in people with D/ID various other techniques and body regions have also been evaluated. These measurements include ultrasounds of the heel [26–28], peripheral DXA of the finger [24], photon absorptiometry of the lumbar sacral spine [22], and central DXA of the femur [23]. These various techniques were not tolerated well in all of the individuals however, due to anatomic deformities or behavioral alterations. For example, 222 of 562 subjects could not have BMD measured due to anatomic deformities or noncooperation [24], and it is likely therefore that different measurement techniques of BMD will be needed in different groups of people with D/ID, depending on their health status.

4. Bone Fractures in the Adult D/ID Population

4.1. Bone Fracture Prevalence. In addition to low BMD, adults with D/ID are also at risk for bone fractures. As with the normal aging population with a disability, fracture detection can be challenging and can be delayed in this population due to profound cognitive, skeletal, and expressive disabilities that prevent the individual from reporting the fracture event or associated pain [29]. Therefore, when caring for these individuals, joint pain should also be factored into the differential diagnosis if an individual is acting out of character [30]. In one study, it was found that adults with D/ID, who were exhibiting destructive behaviors, demonstrated an increased risk of falls and fractures [31]. While such destructive behavior may have been the cause of the fall and subsequent fracture, it may also have been due to the pain from a fracture. A chart review of 994 residents investigated the fracture rate during a 3.5-year period. In this report 182 bones were fractured, giving a fracture rate of 5.2 fractures per 100 persons/year [32], compared to 3 fractures per 100 persons/year in the US civilian noninstitutionalized population from 1980 to 1981 [33]. A review of accident and radiograph reports and institutional registries of 553 individuals with D/ID were reviewed to determine an accurate fracture rate for adults with D/ID. Fracture rates over a 10-month period were compared to all residents of an institution who did not suffer a fracture during the 10-month study period. The mean age of the case participants was 46 years, versus the control participant mean age of 51 years. It was found that 61 fractures occurred among 55 adult residents with D/ID, giving an annual rate of 13.2 fractures per 100 persons/year in the institutionalized adults with D/ID. Men aged 45 to 64 had a higher fracture rate than those 65 and older and those 44 and younger. Women 65 and older had an increased fracture rate, but the difference was not statistically significant [33].

4.2. Fracture Risk Factors. Common fall-related risk factors for fractures in the normal aging population include more than one fall in the past year, the use of psychoactive drugs, a low level of daily living activities before the current fracture, articular symptoms, impaired vision, urinary incontinence, Parkinson's disease [18], and immobility [34]. As with the normal aging population, the level of activity/mobility and antipsychotic medications were risk factors that were assessed in the adults with D/ID. Unique risk factors assessed in the adults with D/ID included level of intellectual disability, antiepileptic use, and epilepsy. Immobility, on the other hand, was found to be a risk factor for recurrent fractures in the general aging population [34], while in the people with D/ID immobility was assessed as a risk factor for first-time fractures. Although not all studies were in agreement [35], overall, the trend was that ambulators seemed to be more at risk for falls than nonambulators [29, 31–33]. For example, the risk of fracture among residents who were independent ambulators was found to be 2.5 times higher than residents who were immobile [33].

4.3. Fracture Sites. Specific fracture sites in people with D/ID have been investigated. In one study, fracture data from a 23-year longitudinal cohort registry of 1434 people with severe and profound developmental disabilities identified that 85% of all fractures involved the extremities and the femoral shaft [29]. There are likely to be two general fracture mechanisms in individuals with D/ID: one, which is largely associated with a lack of weight bearing in people with the least mobility, exemplified by femoral fractures during nontraumatic events (e.g., diapering or transfers); the other, probably due to movement- or fall-related trauma, which is exemplified by hand/foot fractures in people who ambulate [29]. A chart review of 994 adult residents with D/ID revealed that falls were related to 41 (23%) of the fractures, and the cause was indeterminable in 105 (58%). It is likely that the indeterminable fractures were due to patient care such as transfers or bathing [29]. Additionally, hand and foot fractures were also found to be common in this population [32, 36]. This was in accordance with a previous study, which showed that 52% of their individuals with D/ID had fractures of the hands and feet (especially for those under age 65) [33]. In contrast, Glick and colleagues (2005) [29] found that femoral shaft fractures decreased with age while hand/foot fractures increased with age [29].

5. Prevention and Treatment of Low BMD in Adults with D/ID

This section is not intended to be an exhaustive discussion of all the possible prevention and treatment strategies. Rather, the purpose is to introduce a few studies and considerations. A systematic review investigated 6 randomized control trials and 2 controlled clinical trials that investigated the efficacy of various treatments for low BMD in children and adolescents with CP. One of the three bisphosphonate trials showed a large and significant effect on BMD of the femur; one of three weight bearing studies also revealed a large and significant effect but on the lumbar spine [37]. A pilot study demonstrated that 18 months of growth hormone therapy was associated with a statistically significant improvement in spinal BMD [38]. Although numerous studies such as these have investigated treatment of low BMD in children [37–40], relatively few studies have included adults. Since vitamin D levels are essential for normal skeletal mineralization and bone metabolism, vitamin D status was evaluated in institutionalized adults with ID in an open label trial [41]. This study compared oral and intramuscular (IM) administration of vitamin D in 138 Finnish adults with ID. Currently, IM vitamin D is rarely utilized to treat low BMD. Baseline serum 25-OH-vitamin D, calcium, phosphate, alkaline phosphatase, and parathyroid hormone (PTH) levels were measured. The cohort was divided into two treatment groups: one group received vitamin D3 (800 IU) orally per day; the other group received a single vitamin D3 dose (150,000 IU) as an intramuscular injection. All participants were also administered calcium orally (1000 mg per day). Serum 25-OHD levels were measured again at 6 months after the onset of starting the vitamin D and

calcium regimen. The gender distribution and proportion of participants on antiepileptics were similar in both groups. None of the participants were taking vitamin D or calcium supplements at baseline, and the majority (65%) of patients were ambulatory. At baseline, there were no significant differences in biochemical values between the two groups, and the means for 25-OH vitamin D were 40 nmol/L and 41 nmol/L, respectively. These low vitamin D levels were associated with secondary hyperparathyroidism in 17% of the patients. In the group that received oral vitamin D, the mean S-25-OHD increased to 82 nmol/L. In the group that received vitamin D through intramuscular injection, the mean S-25-OH increased to 62 nmol/L. The plasma PTH levels decreased in both groups. This study demonstrated that vitamin D insufficiency is common in adults with ID and that either oral or intramuscular administration of vitamin D can increase mean S-25-OHD levels without adverse effects [41].

A treatment study was recently conducted to determine the safety and efficacy of teriparatide (a recombinant form of PTH) in nonambulatory institutionalized men and women with osteoporosis [42]. In this trial, bone biomarkers were used to assess efficacy. Teriparatide (20 µg subcutaneous) was administered daily for up to 18 months at one institute and 24 months at the other [42]. All participants received at least 400 IU vitamin D orally a day and at least 1000 mg/day of calcium. Markers of bone formation (procollagen type 1 intact N-terminal propeptide (PINP)) and resorption (C-telopeptide (CTx)) were measured at 3-month intervals. Serum calcium was measured at 2 week intervals for 12 weeks and thereafter at 3-month intervals, and 27 individuals received at least one injection. The incidence of hypercalcemia was 11.1% and led to medication discontinuation in one participant. Biomarkers of bone formation increased, doubling by three months. At 12 months, PINP and CTx levels remained elevated from baseline. It was concluded that teriparatide was safe and effective in this population, however serial calcium measurements are recommended, especially during the first 3 months. It has been suggested that bisphosphonates necessitate maintaining an upright posture for at least 30 minutes after taking them, which may be difficult for some D/ID individuals [31]. Also, the risk of esophageal ulceration may be increased in individuals with D/ID whose disabilities include oral motor dysfunction, which is particularly a concern if the individual is unable to communicate the pain [31].

6. BMD and Down Syndrome (DS)

DS, trisomy of chromosome 21, is the most common identified cause of severe intellectual disability. In addition to intellectual disability, it is associated with cardiac, endocrine, gastrointestinal, skin, hearing, and vision dysfunction, and growth failure [43]. The Centers for Disease Control and Prevention (CDC) estimates that each year approximately 6,000 babies in the United States are born with Down syndrome (1 of every 691 births) [1]. In order to try to understand the generation of abnormal BMD in the DS

and CP populations, studies that include children will also be included in our paper. In a small study, the BMD of 10 Chinese children with DS (7 boys, 3 girls, aged 10–16 years) was compared to 10 age-matched controls. The BMD of the 2nd to 4th lumbar vertebrae was measured using a dual photon absorptiometer. The BMD of the DS patients' values ranged from 0.65 gc-2 in the 10-year olds to 1.00 gc-2 in the 16-year olds and was reportedly lower than the controls. The BMD of the DS children increased from age 10 to 16 years, but was reportedly significantly lower than in normal children of the same age group [44]. A more recent cross-sectional study [45] utilized a larger sample size of children and adolescents with DS and corroborated the results by Kao et al. [44]. Thirty-two children and adolescents (15 females and 17 males) with DS between 10 and 19 years were compared to an age-matched sample of 32 healthy subjects (13 females, 19 males) without DS. Bone mass at the lumbar spine (L1–L4) and femur (hip and femoral neck) was measured with DXA using a pediatric version of the software. Volumetric BMD (vBMD) was estimated for the lumbar spine and femoral neck using simple geometric cylindrical models. BMD/height (BMDH) was calculated to adjust bone mass for whole body bone size. No results were provided for specific ages within this cohort, however. After adjusting the raw values by Tanner stage (a scale used to describe the onset and progression of pubertal changes) height, and total lean mass, it was found that females with DS, compared to females without DS, had lower bone mass density (0.085 ± 0.008 versus 0.092 ± 0.004) and BMD/height (0.61 ± 0.04 versus 0.64 ± 0.01). DS males and females had lower BMD in the whole body compared to the controls (males: 0.928 ± 0.127 DS versus 1.049 ± 0.128 controls, females: 0.845 ± 0.086 DS versus 1.014 ± 0.109 controls). DS females had lower lumbar spine BMD than the DS males (0.76 ± 0.118 versus 0.788 ± 0.146) [45]. Baptista and colleagues also compared bone mineral mass adjusted for bone and body size in limbs, lumbar spine, and the femoral neck but did not find significant findings in their subjects that were less than 20 years [46]. Their subjects consisted of 66 females (33 with DS) and 68 males (34 with DS) aged 14–40 years, living in the community. DXA was used to measure BMD, and volumetric BMD and femoral neck strength were calculated. DS was shown to be a risk factor for both low lumbar spine volumetric bone mineral density and femoral neck strength in the group older than 20 years old, compared to adolescents. In another study, Guijarro and colleagues also investigated the impact of short stature of DS patients on bone mass [47]. Their assessment involved 39 ambulatory patients with DS (18 male, 21 female) ranging in age from 18 to 45 (mean age of 26) and age-matched controls. A DXA was used to assess BMD of the spine, hip, and the total body and percentage of fat and lean mass, and the volume of BMD at the lumbar spine and femoral neck was computed. A reduced BMD was found in all DS patients in the spine, hip, and total body. Spine volume BMD was also lower in DS than controls (0.140 versus 0.149 g/cm^2) [47]. The main causes of low BMD in the DS population are likely to include endocrine abnormalities such as thyroid and gonadal dysfunction, reduced physical activity, hypotonia

(low muscle tone), and reduced muscle strength [22, 47, 48]. It has also been suggested that the distal region of chromosome 21 may be associated with osteoporosis [49]. Although men with ID with and without DS have been shown to have lower quadriceps muscle strength, only DS males demonstrated lower spine BMD than the healthy males of the control group [48]. The limited studies to date suggest that although DS children as young as ten years old have abnormal BMD it is not until after about age 18 that the BMD was found to decrease. However, limited *T* and *z* scores were included in the results.

7. BMD and Cerebral Palsy (CP)

CP is the most common motor disability in childhood, and the CDC estimates that an average of 1 in 303 children in the USA are diagnosed with CP [1]. CP consists of a group of permanent disorders of movement and posture development, causing activity limitations, which are attributed to nonprogressive disturbances that occurred in the developing fetal or infant brain. The motor disorders of CP are often accompanied by disturbances in sensation, perception, cognition, communication, and behavior, by epilepsy and by secondary musculoskeletal problems. The range of movement limitation is variable and may range from independent ambulation to an inability to raise one's head. A more detailed discussion of the classification of cerebral palsy was recently reviewed [50].

Over the past thirty years, various studies have investigated BMD and fractures in children with moderate to severe CP. Recently, a systematic review was completed that identified a limited amount of high-quality evidence on low BMD and fractures in children with severe CP [51]. Fractures are common in individuals with moderate to severe CP, with an incidence of approximately 4% per year [52], compared to approximately 2.5% in healthy children [51]. Low BMD is a serious problem in children with moderate to severe CP. Clinical *z* scores have been found to range from -3.4 in the distal femur to -0.8 in the lumbar spine [53–55]. There has also been shown to be a relationship between advancing age and declining BMD *z* scores at distal femur sites. Ages 2 to 5.9 had a *z* score of -2.9 ± 0.4 , ages 6–11.9 had a *z* score of -3.0 ± 0.2 , and ages 12 to 19 had a *z* score of -3.7 ± 0.3 . It was found that 71 boys had a mean *z* score of -3.1 ± 0.2 of the distal femur and 46 females had a mean *z* score of -3.6 ± 0.3 of the distal femur [53]. These similar *z* scores of the distal femur of the same age groups were also replicated in a more recent study by Henderson and colleagues [55]. Additionally, the BMD of the radius and tibia was measured in 45 children and young adults with moderate to severe CP by ultrasound. The *z* scores for the radius were from ages 1 to 10 -0.8 ± 1.1 , ages 11–20 -1.0 ± 1.4 , and ages 21–29 -1.1 ± 1.1 . The females had a mean *z* score of -1.4 ± 1.3 and males -0.6 ± 1.1 [56]. The former two studies [53, 54] measured the BMD of the femur, which is the most commonly fractured in this population, with a DXA; the latter study on the other hand used ultrasound to measure the radius. The prevalence of low BMD of the distal femur measured by DXA (defined as *z* score lower than -2) was 77% in

a population of 117 children aged 2–19 with moderate to severe CP. The most commonly studied determinants of low BMD were gross motor function classification system (GMFCS) level, feeding difficulties, previous fracture [51, 53], and the use of antiepileptic drugs [57, 58]. Severity of neurologic impairment measured by GMFCS, increasing difficulty feeding the child, use of antiepileptics, and lower triceps skinfold all independently contributed to lower BMD *z* scores in the femur (in decreasing order of importance) [53]. It was accepted that absence of weight bearing is an important direct cause of low BMD in children with CP; however, it was found that BMD *z* scores were significantly lower in GMFCS level 5 children than in level 4 children, yet both groups are nonambulatory [53]. Based on these studies, there seems to be a trend towards increasing age and low BMD in CP. Uniquely, the low BMD has been found in children as young as age 2.

Anthropometric and fitness variables were also recently assessed to determine if they would be useful for detecting children with potentially reduced bone density. Growth variables were mainly related to femoral and lumbar bone densities, while muscular endurance was mainly related to femoral and calcaneus bone densities. This suggests that multiple complex variables can contribute to bone density variations among different skeletal areas in these children [59]. Results from another recent study suggest that muscle strength, especially antigravity muscle strength, was more associated with the bone density of ambulatory children with CP than motor function [60]. However, it must be noted that not all predictive factors for developing low BMD in this population have been studied; for example, daylight exposure time and amount of exercise have not been investigated [51].

A limited number of studies have investigated the bone health of adults with CP [57, 61]. In the Nakano et al. study, 123 institutionalized adults with CP (mean age of men was 31.5 years and mean age of women was 33.3 years) had the BMD of their 2nd metacarpal bone measured by a hand absorptiometer [61]. Although no actual *T* or *z* scores were provided in this study, the authors reported that the study subjects had poor bone health. Ambulation was significantly associated with higher BMD in women, and hypocalcaemia, hypophosphatemia, and elevated alkaline phosphatase levels were found in 28% of the men and 31% of the women [61]. In the King et al. study [57], it was investigated whether children and adults with spastic quadriplegic CP, who are nonambulatory, would have lower BMD that worsens with age when compared to age- and gender-matched controls. In this study 51 participants were recruited from institutions and community settings. BMD was measured with DXA, skeletal surveys were performed to assess fractures, and biochemical analyses including calcium, phosphorous, magnesium, 25-OH vitamin D, and osteocalcin were performed. Participants ranged from 5 to 48 years of age. The mean *z* score for the lumbar spine for all study participants was -2.37 ± 0.21 . When the participants were divided into groups of 18 years or less and greater than 18 years, the *z* scores were similar; for those 18 years or less the mean *z* score was $-2.32 \pm 0.23 \text{ gm/cm}^2$ ($n = 30$)

versus -2.45 ± 0.40 for those greater than age 18 years. Interestingly, no correlation was found between BMD *z* score and age in this study. There were no differences in BMD *z* score between all participants when corrected for bone age versus uncorrected [57].

8. Conclusions

Many studies have demonstrated that BMD is decreased in both men and women with D/ID, and especially those with DS and CP. Some of the risk factors for decreased BMD in D/ID include endocrine abnormalities such as thyroid and gonadal dysfunction, reduced physical activity, hypotonia and reduced muscle strength, vitamin D deficiency, and certain medications. It is presently unclear whether the bone mass decrease is similar to that seen in populations without D/ID or whether it is the continuation of a process begun in early childhood, and future studies will be needed to address this important topic. The DXA scan is currently the standard measurement technique of BMD of the hip and lumbar spine in the general population, but in the D/ID population these specific measurements are not always feasible to collect. Thus, it is also clear that one size does not fit all with regard to measurement techniques of BMD in people with D/ID and future studies are needed that thoughtfully and creatively identify appropriate tools for measuring the BMD in this population. Measurements taking into account body and bone size (by calculating an adjusted BMD) as like was done for individuals with DS may need to be replicated for other syndromes or endocrine diagnosis that include short stature such as Turner syndrome, Rett syndrome, or growth deficiency.

Although various genetic and environmental risk factors for low BMD in individuals with D/ID have been identified, the exact molecular mechanisms underlying the low bone density have yet to be elucidated. Until the mechanisms for this diverse population are identified it is difficult to begin to propose logical nonpharmacologic or pharmacologic interventions. One low BMD prevention study and one treatment study for this population utilized serum biomarkers as an outcome marker. It would be practical and clinically relevant to take the outcome measurement a step further and to investigate improved repeat BMD and/or overall reduction in fractures. Independent of low BMD, clearly this population is at risk for fractures. The majority of the studies were in agreement that the fracture rate increased with mobility and that hand and feet fractures were the most common, although a large proportion of the populations had fractures without a known cause. This paper has specifically focused on discussing D/ID, DS, and CP in relation to BMD. However, BMD is only one measure that can be used to assess a very complex structure such as bone. Several studies have found differences in other bone health variables in genetic syndromes [62, 63], and children with CP (even as young as 2–5 years old) have been found to have low BMD. This raises the question of the direct effects of gene mutations or polymorphisms on bone cellular processes [64] or secondary effects due to neurological or structural impairments that affect bone health [62]. For example,

individuals with the developmental disorder Prader-Willi (PW) syndrome are known to develop osteoporosis [65]. This is thought to be caused by an abnormal expression of genes within the PW-critical region on bone cellular functions and is secondary to associated findings, such as hormonal imbalances and hypotonia [62]. It is clear that further work is needed to unravel these complex findings, and gaining a greater understanding of changes in BMD in people with D/ID could lead to the development of more advanced diagnostics and treatments for bone loss in this population.

Abbreviations

ID:	Intellectual disability
DD:	Developmental disability
DS:	Down syndrome
CP:	Cerebral palsy
BMD:	Bone mass density
vBMD:	Volumetric bone mass density
DXA:	Dual-emission X-ray absorptiometry
US:	Ultrasound
S-25-OH:	Serum 25 hydroxyvitamin D
PTH:	Parathyroid hormone
CDC:	Centers for Disease Control and Prevention.

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Review Article

Central Role of the EGF Receptor in Neurometabolic Aging

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A strong connection between neuronal and metabolic health has been revealed in recent years. It appears that both normal and pathophysiological aging, as well as neurodegenerative disorders, are all profoundly influenced by this “neurometabolic” interface, that is, communication between the brain and metabolic organs. An important aspect of this “neurometabolic” axis that needs to be investigated involves an elucidation of molecular factors that knit these two functional signaling domains, neuronal and metabolic, together. This paper attempts to identify and discuss a potential keystone signaling factor in this “neurometabolic” axis, that is, the epidermal growth factor receptor (EGFR). The EGFR has been previously demonstrated to act as a signaling nexus for many ligand signaling modalities and cellular stressors, for example, radiation and oxidative radicals, linked to aging and degeneration. The EGFR is expressed in a wide variety of cells/tissues that pertain to the coordinated regulation of neurometabolic activity. EGFR signaling has been highlighted directly or indirectly in a spectrum of neurometabolic conditions, for example, metabolic syndrome, diabetes, Alzheimer’s disease, cancer, and cardiorespiratory function. Understanding the positioning of the EGFR within the neurometabolic domain will enhance our appreciation of the ability of this receptor system to underpin highly complex physiological paradigms such as aging and neurodegeneration.

1. Introduction

The epidermal growth factor receptor (EGFR) is a 170-kDa single-pass transmembrane (TM) protein. The EGFR belongs to a family of four members: erbB-1/EGFR, erbB2 (HER2/c-neu), erbB3 (Her3), and erbB4 (Her4). The basic EGFR structure contains an immunoglobulin-like extracellular ligand binding domain and an intrinsic tyrosine (Tyr) kinase activity contained within its intracellular carboxyl terminal domain. Extracellular stimulating ligands, for example, epidermal growth factor (EGF), bind to an individual TM receptor and induce a conformational alteration that causes dimerization with another ligand-bound EGFR or with another erbB receptor. The ligand-induced conformational changes then activate the intrinsic tyrosine kinase domains causing subsequent autophosphorylation or transphosphorylation of the opposing receptor in the dimer [1, 2]. The creation of phosphorylated tyrosines then serves to create a dynamic scaffolding domain for downstream

signaling molecules that possess SH2 or PTB domains, for example, Grb2 or Shc. Not only are positive signaling molecules recruited in this manner but also factors involved in EGFR internalization, such as Eps15 [3], and eventual lysosomal degradation factors, such as c-Cbl [4, 5]. Many of the signaling functions of the EGFR are mirrored by other members of the receptor tyrosine kinase (RTK) receptor class, for example, ligand-induced tyrosine kinase activation, protein scaffold assembly, and interaction with multiple common downstream factors (phospholipases, phosphoinositide kinases, non-RTKs) [6]. The commonality of function between RTKs indicates that these receptors are highly likely to form important, and strongly interconnected, links between a diverse range of physiological or pathophysiological activities.

1.1. EGFR Activation Profile Diversity. The activation process of the EGFR typically follows the generic process of EGFR ligand stimulation, tyrosine kinase activation, dimerization

and tyrosine (Tyr) phosphorylation, and then signaling protein complex assembly. However, there are considerable functional nuances within this process that serve to profoundly condition and add “*texture*” to the signaling output from these important receptors. For example, the specific nature of the carboxyl terminal domain phosphorylation events provides a mechanism by which a greater variety of signaling information can be conveyed to the intracellular *milieu*. Tyr phosphorylation for EGFR may occur at various sites including Tyr 845, 992, 1045, 1068, 1148, 1173, or 1086. Phosphorylated Tyr residues act as binding sites for proteins containing Src-homology 2 domains (SH2) such as Grb2, Shc and phospholipase C-gamma (PLC- γ). Phosphorylation at Tyr 845 in the kinase domain is involved in stabilizing the activation loop, maintaining the active state enzyme, and providing a binding surface for substrate proteins [7, 8]. The non-RTK c-Src is also involved in EGFR phosphorylation at Tyr 845 [9]. The SH2 domain of PLC- γ binds at phospho-Tyr 992, which results in activation of PLC- γ -mediated downstream signaling [10]. A pair of phosphorylated EGFR residues (Tyr 1148 and Tyr 1173) provides a docking site for the Shc scaffold protein, and both sites are involved in mitogen activated protein kinase (MAPK) signaling activation [1]. Phosphorylation of EGFR at Tyr 1045 creates a major docking site for the adapter protein c-Cbl, leading to receptor ubiquitination and degradation following EGFR activation [4, 5]. The multifunctional Grb2 adapter protein binds the EGFR at phospho-Tyr1068 once the EGFR is activated [11]. Phosphorylation of the EGFR at specific serine (Ser) and threonine residues inhibits EGFR kinase activity. EGFR carboxy-terminal residues Ser1046 and Ser1047 are phosphorylated by CaM kinase II; mutation of either of these serines results in upregulated EGFR tyrosine autophosphorylation [12]. Therefore the pattern and timing of such EGFR posttranslational phosphorylation events strongly affect its cellular disposition and eventual signaling capacity. Such a wide flexibility of function is typically characteristic of protein factors that are likely to form multidimensional interactions within more complex physiological paradigms [13, 14].

1.2. Diversity of EGFR-Stimulatory Factors. The EGFR possesses a highly complex relationship with its stimulating ligands/factors. Endogenous peptide-based ligands that can activate the EGFR include epidermal growth factor (EGF), transforming growth factor (TGF)- α , amphiregulin (AR), heparin-binding EGF (HB-EGF), betacellulin, epiregulin (ER), and epigen [15]. The EGFR is almost unique amongst receptor systems in that it serves as a molecular integration site for multiple types of stimuli including: peptide ligands, metal ions, ultraviolet and gamma radiation, osmotic shock, membrane depolarization, and oxidative radicals [16]. This wide range of stimuli again reinforces the concept that the EGFR acts as a functional keystone in “higher-order” complex systems that may underpin multifactorial global somatic actions such as aging and metabolism.

The EGFR can be activated by small transition metal ions such as zinc and copper (Cu) [17]. In addition to

simple metal ions, ultraviolet (UV) radiation activates the EGFR by two ligand-binding independent mechanisms: Tyr phosphorylation of the receptor [18–20], and via oxidative inhibition of the RPTP- κ [21]. It appears that the generation of reactive oxygen species (ROS) may in some cases be secondary to EGFR activation. These ROS may lead to the reversible inactivation of a crucial protein for EGFR activation, that is, protein tyrosine phosphatases (PTPs) by oxidizing the catalytic cysteine in their active site [22, 23].

Heptahelical G-protein-coupled receptors (GPCRs), which classically were not primarily associated with tyrosine kinase signaling cascades, have subsequently been demonstrated to exert a profound regulatory capacity over RTK systems, including the EGFR [24–29]. This GPCR-RTK regulatory capacity has been demonstrated for multiple types of GPCRs, for example, muscarinic, bombesin, thrombin, endothelin, lysophosphatidic acid (LPA) receptors, which perhaps suggests that this is a common function for GPCRs in most physiological processes. There appear to be multiple mechanisms that can mediate this functional GPCR-RTK interaction, however one of the most studied involves the GPCR-induced processing of pro-HB-EGF to its soluble form through the activation of intramembrane metalloproteinases [30]. Matrix metalloproteinases (MMPs) shed the pro-form of the EGFR ligands which are then locally liberated and able to induce classical EGFR activation. It is interesting to note that this so-called G protein-coupled receptor RTK “transactivation” is a process not only limited to heptahelical GPCRs. For example, the insulin-like growth factor-1 receptor (IGF-1R), classically considered to possess an RTK structure, is actually a “*functional G protein-coupled receptor*” as it is able to stimulate guanine nucleotide exchange event within its associated G proteins [31]. IGF-1-mediated stimulation of the IGF-1R can therefore result in a “transactivation” of the EGFR in a manner similar to that induced by heptahelical GPCR activity [32]. This interaction between the IGF-1R and the EGFR perhaps forms one of the first points of molecular integration between neurological and metabolic activities. In addition to this functional system bridge between neuronal and metabolic systems, ligands of G α q/11-coupled GPCRs that are associated with insulin resistance, such as serotonin, endothelin-1, and thrombin, may also stimulate HB-EGF production and transactivate EGFR in 3T3-L1 adipocytes. Serotonin has been shown to additionally have this effect in primary adipocytes and myotubes [33]. Not only can serotonin induce activation of the EGFR but it also appears to be able to regulate the posttranslational activity, via serine phosphorylation and the mTOR pathway, of the insulin/IGF-1 receptor-associated protein, IRS-1 [33]. IRS-1 and -2 are insulin receptor-associated scaffold proteins essential for effective glucose metabolism in multiple energy-regulatory tissues such as the liver [34]. This serotonergic synergy between IRS-1 phosphorylation and HB-EGF-mediated EGFR stimulation has been demonstrated to play a key role in serotonin-induced insulin resistance [35]. This molecular linkage may underpin the ability of antiserotonergic therapy amelioration of glucose tolerance in diabetics [36]. Insulin resistance has been shown to

have causal relationships with proinflammatory cytokines (including adipokines) [37], prolonged/amplified insulin stimulation, oxidative stress, and endoplasmic reticulum stress [38–40]. Indicating the significant interaction of the EGFR system with other signaling modalities, it has been shown that activation of the growth hormone cytokine receptor can induce phosphorylation of EGFR in preadipocytic fibroblast cell lines. This cytokine receptor-mediated transactivation event effectively modulates EGF-induced EGFR trafficking and signaling [41]. It is important to note that the growth hormone ligand and receptor system is one of the most important factors that controls both IGF-1 expression and secretion as well as somatic energy metabolism.

It has been revealed in recent years that the Toll-like receptor (TLR) system is important for neurological health [42]. Ligand activation of multiple TLR isoforms has been demonstrated to induce subsequent activation of EGFRs [43, 44]. This pathway involves the activation of nicotinamide adenine dinucleotide phosphate and the generation of reactive oxidative species (ROS). Subsequent activation of tumor necrosis factor (TNF)- α -converting enzyme (TACE) leads to the liberation of TGF- α from the epithelium and productive ligation of the EGFR [45]. TLR2 activation by lipoteichoic acid leads to a disintegrin and metalloproteinase (ADAM)-10-induced cleavage of HB-EGF, another EGFR ligand. Amphiregulin has also been shown to be released in response to TNF- α [46].

It is clear therefore that in addition to its widespread expression and highly textured signaling activity, the EGFR is subject to multiple and highly diverse signaling inputs both from other receptor systems and small molecules and ions. These combined molecular factors and emerging evidence of functional overlap of EGFR activities from neuronal to metabolic systems suggest that the EGFR system may exert a “near omnipotent” function in aging biology.

2. EGFR and the Control of Neurometabolic Physiology

The EGFR, and its axis of ligands demonstrate a widespread expression profile across the central nervous system (CNS) [47]. Substantial EGFR expression is found in the neocortex and limbic cortex, cerebellum, cerebrovascular endothelial cells [48, 49], and the midbrain [50]. EGFR has been detected in the hippocampal pyramidal cells, Purkinje cells, large multipolar neurons of the dentate nucleus, anterior horn cells, dorsal root ganglion cells, cells of the dorsal nucleus of Clark, intermediolateral column cells, and ependymal cells [51]. In the human fetal brain, EGFR has been shown to be expressed in the subventricular zone (SVZ) [52], hippocampus, and cerebellum [53]. This expression pattern in the two primary areas of adult neurogenesis suggests that the EGFR could also play a strong role in age-related neuronal survival and regeneration. The EGFR has also been implicated in chemotactic migration in the developing telencephalon with implication of HB-EGF involvement [54]. EGF appears to act as a mitogen for neural stem and

progenitor cells (NS/NPCs) in the CNS as well as numerous other cell types involved in neurometabolic activity. Along with EGF, HB-EGF and TGF- α also promote proliferation in the SVZ of an adult mouse HB-EGF [55]. *In vitro*, EGF maintains NS/NPCs in the proliferative state, whereas in the normal rodent brain, it induces proliferation and migration in the SVZ. EGF can also increase neuronal replacement in the ischemia-injured adult striatum. In addition, Sun et al. (2010) found that EGF is neuroprotective rather than neurogenic when protecting the brain from injury [56].

Notch proteins are a family of transmembrane receptor proteins with repeated extracellular EGF and titular Notch domains. Notch interacts with cell-bound ligands (*Delta-like*, *Jagged*) that facilitate an inter-cellular signaling pathway that is critical in cellular and tissue development. Notch family members regulate developmental processes by controlling cell fate decisions [57]. Notch and EGFR have fundamental and selective roles in the maintenance of NS/NPCs in the SVZ. Notch signaling promotes proliferative signaling during neurogenesis and its activity is inhibited by *Numb* to promote neural differentiation. The *Numb* gene product controls binary cell fate decisions in peripheral and central nervous systems during neurogenesis. Notch and EGFR pathway interaction regulates neural stem cell number and self-renewal [58]. Altering particular signaling mechanisms in selective cell types of the SVZ can cause profound changes in the overall cell composition of this neurogenic region of the adult brain. Defining interactions and homeostatic mechanisms that occur between different types of SVZ cells under normal conditions provides crucial information on possible alterations of specific signaling pathways that might occur under pathological conditions or after brain injury.

The aspartyl protease, γ -secretase, is a multisubunit protease which mediates the coordinated intramembrane proteolysis of both Notch and amyloid-precursor protein (APP), which are both implicated in the etiology of Alzheimer's disease (AD). In some cell types the expression of EGFR and γ -secretase have been reported to be inversely related to each other, suggesting the existence of strong functional connection between these two systems [59]. In a study of squamous cell carcinoma, Notch and EGFR were shown to participate in the tumor suppressor function of γ -secretase, again reinforcing the existence of a physiologically-relevant EGFR- γ -secretase interaction [60].

Given the considerable evidence for a strong role of the EGFR in neural stem cell development it is unsurprising that one of the functional sequelae of this activity is the maintenance of memory pattern formation in the hippocampus. Two of the most important regions of the CNS for adult stem cell-mediated neurogenesis are the hippocampal CA1 and dentate gyrus (DG) [61]. Membrane-tethered pro-ligand, HB-EGF, is found in cells within many regions of the CNS, for example, cerebellum, cerebral cortex as well as the hippocampus [62]. The specific hippocampal expression of HB-EGF and the association of the EGFR with neurodevelopmental processes have led to the implication of EGFR signaling in synaptic plasticity and memory formation [55]. HB-EGF knockout mice demonstrate also reduced CNS

expression levels of neurotrophic factors including brain-derived neurotrophic factor (BDNF), which is a ligand for the tropomyosin-receptor kinase- (Trk-) B receptor [55]. Expression levels of BDNF and TrkB receptor activity are both strongly correlated to the regulation of synaptic architecture and the ability to form and retain memory patterns. The hippocampal regions, CA1/DG, demonstrate a tremendously high requirement for energetic support due to their constant stimulation both from sensory inputs and higher areas of the brain. Their huge energetic load therefore makes CA1/DG neurons extremely sensitive to the metabolic status of the individual [63–65].

One of the strongest points of physiological neurometabolic intersection may occur via the neurometabolic control of reproductive activity. It has been demonstrated that there are strong gender-specific molecular mechanisms that link hippocampal cognitive function to centrally-controlled reproductive behavior [66–69]. It has also been demonstrated that the EGFR is localized to the anterior pituitary [70] along with EGF [71] and TGF- α [72], implicating them in the reproductive hypothalamic-pituitary axis [73]. In the anterior pituitary, EGFR has been identified in lactosomatotrophs [74], corticotrophs [75], and gonadotrophs [76, 77]. The EGFR has been directly implicated in corticotroph proliferation and hormone secretion, while TGF- α has been implicated in EGFR-dependent estrogen-mediated corticotroph cell proliferation [78, 79]. In addition to these reproductive functions, EGFR activity is also closely associated with the dynamic regulation of prolactin transcription and synthesis [80].

3. Role of the EGFR in Neurometabolic Pathophysiology

As one would expect from its multifactorial biological activity, the genomic inactivation of EGFR by homologous recombination results in profound systemic effects. EGFR genomic inactivation can induce three different phenotypes that range from peri-implantation lethality to postnatal lethality [81]. EGFR-inactivated mice die at different stages of development depending on their genetic background; EGFR mutant mice may die at gestation (129/Sv), at birth (C57Bl/6), or they may live up to 8 or 20 days (CD1, MF1, 129Sv/J Swiss Black or C3H) [82–86]. After parturition, the surviving animals may suffer from impaired epithelial development in organs such as the skin, lung and gastrointestinal tract [83] as well as the placenta. Furthermore, there is a strain-independent postnatal neurodegeneration in the frontal cortex, olfactory bulb, and thalamus in surviving EGFR-null mice [82]. This neurodegeneration is characterized by massive apoptosis and upregulation of *c-fos* [82]. The mildest form of EGFR inactivation leads to epithelial immaturity and postnatal death due to respiratory failure and necrotizing enterocolitis-like lesions in the intestine [81]. The defects seen in this “postnatal lethality phenotype” manifest in the classical EGF-responsive organs (skin, intestine) and organs undergoing branching morphogenesis during development (lung, kidney, mammary gland, pancreas, and

prostate) and, thus, are in concordance with the concept of EGF family members being important epithelial mitogens. Intestinal changes observed in the EGFR-inactivated mice differ in the level of severity, the endpoint being severe mucosal lesions and necroses (cell death) [81].

Within the CNS, it has been suggested that the EGFR is likely to mediate the effects of EGF/TGF- α on neuronal differentiation [87], survival [88–90], as well as glial proliferation [91, 92]. While many neurons of the CNS constitutively express the EGFR [48, 49], glia and endothelial cells demonstrate induced receptor expression following acute injury or chronic neurodegeneration [48, 49, 93–95]. Glial cells are vital for the generation of a dynamic support structure for CNS activity in the face of altered energy availability or oxidative stress [96]. TGF- α -gene expression in glial cells is a component of the hypothalamic response to injury [97] and it has been reported that during development, the increased bias towards glial differentiation is not dependent on EGFR signaling [98]. EGFR expression appears to be crucial for the proliferation and differentiation of astrocytes [92], however, it is only one of a number of means by which astrocytes can be induced. Burrows et al. (1997) demonstrated that misexpression of EGFRs promoted astrocyte development prematurely *in vivo* [99]. Furthermore, it was suggested that EGFRs may induce the development of astrocytes by regulating the responses of progenitors to various extrinsic signals such as leukemia inhibitory factor (LIF) and bone morphogenetic proteins (BMPs) [99]. It was also later demonstrated that EGFRs elevate STAT3 expression and increase its phosphorylation by LIF and that EGFRs further regulate LIF downstream of STAT3 but they do not regulate changes in responsiveness to BMPs [100].

Mice that do not possess EGFR develop neurodegeneration involving the frontal cortex and olfactory bulbs. It has been shown that EGFR signaling controls cortical degeneration by the regulation of cortical astrocyte apoptosis [101]. Midbrain astrocytes where EGFR is absent are not affected; however, some mutant cortical astrocytes possess an increased incidence of apoptosis which is mediated by an Akt-caspase-dependent mechanism and these cells demonstrate a reduced ability to support neuronal survival [101]. These results suggest two functionally distinct astrocyte populations exist, which are differentially dependent on EGFR signaling for their survival and also for their ability to support neuronal survival [101]. These spatial differences in astrocyte composition provide a mechanism for the region-specific neurodegeneration in EGFR null mice [101].

TGF- α contributes to the neuroendocrine regulation of female puberty by stimulating the release of luteinizing hormone-releasing hormone (LHRH) [97]. It has been proposed that TGF- α may be involved in a glial-neuronal interaction where it first stimulates PGE2 from glial cells which in turn elicits LHRH from neuronal terminals [102]. Interestingly astrocytes, but not LHRH neurons, express EGFR [103]. Disruption of astroglial EGFR signaling leads to irregular estrous cycles and a decreased secretion of LH in female mice which eventually can cause infertility [104]. The EGFR and TGF- α are present in the suprachiasmatic nucleus

(SCN), which is the core circadian pacemaker. The SCN releases factors acting locally within the hypothalamus, more specifically in the neurons of the subparaventricular zone. It has also been reported that EGFR has a circadian-time-dependent neuromodulatory function in the SCN [105, 106]. TGF- α has been implicated as an inhibitor of locomotion and sleep-wake cycles [107]. In hamsters, these data are confirmed, and, furthermore, it was shown that central administration of TGF- α induced weight loss [108].

4. EGFR and Neurodegenerative Disease

AD is one of the most prevalent familial and sporadic neurodegenerative disorders. One of the strongest risk factors for AD appears to be advancing age. In addition to the clear neurological basis of AD, the contribution of metabolic factors to this disorder as well as other neurological processes has been the subject of considerable recent scrutiny [65, 109–111]. In AD pathology, aggregates and deposits of the protein fragment β -amyloid (plaques) and twisted strands of the protein Tau (neurofibrillary tangles) induce the generation of nerve cell damage and cell death in areas of the brain including the cortex and especially the hippocampus [110, 112–114]. With respect to an association with AD etiology it has been demonstrated that EGFR expression in the rat brain reduces with age both in males and females [115]. AD-related neuritic plaques observed in the cerebral cortex and hippocampus of patients with AD also immunostain positively for EGFR [93]. In addition, it has been demonstrated that EGFR expression is upregulated in astrocytic cells in AD [116] and that EGFR-null astrocytes from mutant cortices in mice have an impaired proliferative capacity both *in vitro* and *in vivo* [82, 86]. With respect to the potential involvement of the EGFR in the etiology neurometabolic pathophysiology, such as AD, a strong role for metal ion activity is likely. It has been demonstrated that AD is often associated with a central nervous imbalance of transition metal levels which may lead to neurotoxicity. Transition metals facilitate oxygen transport and its eventual usage by nervous tissue. The same metal ion chemistry if dysregulated, for example, Cu imbalance, can induce the generation of reactive oxygen species (ROS) which are strongly associated with AD and pathological aging [117]. Interestingly, clioquinol-Cu metal complexes, which are cell-permeable, can be employed to ameliorate AD pathology via their ability to stimulate the EGFR in neuronal-derived cell lines causing a reduction in β -amyloid levels [118]. This metal ion process also appears to involve the activation of TM matrix metalloproteinases (MMPs) as well as the nonreceptor tyrosine kinase c-Src [118]. Iron is another metal implicated in AD through its capacity to modulate the ability of amyloid peptides to yield ROS [119] which then may serve to exacerbate the disorder further [120].

EGFR immunoreactivity has been localized to the brain, pituitary, skin vascular endothelial cells of clinically diagnosed, pathologically confirmed AD patients when samples were collected postmortem [95]. It has also been reported that EGFR is localized to the luminal surface of endothelial cells and that EGFR immunoreactivity in brain vasculature

was present in all assessed elderly patients with dementia, when compared to nondemented patients [94]. As microvascular damage and hypertension are strongly associated with intermittent ischemia, hypoxia, and AD-related pathology, it seems likely that the cerebrovascular activity of the EGFR is an important aspect of its role in neurodegenerative pathophysiology. In addition to a potential role in AD, it has been shown that there is an increased expression of EGFRs in the striatum in chronic Parkinsonian syndromes but not in acute models of the disease [121]. Depletion of neuronal dopamine is observed in Parkinson's disease and this subsequently would lead to decreased precursor cell proliferation in the SVZ as well as a reduction in local EGF production. EGFR positive cells have been reported to be depleted in SVZ in Parkinson's disease as well: these data collectively indicate a role of dopamine-EGFR signaling loop in regulation of neurogenesis of dopaminergic neurons [122]. This process appears to involve an Wnt5a-dopamine D2 receptor interaction in association with the EGFR [123]. Taken together there appear to be multiple and mechanistically diverse routes by which the EGFR may be involved in the neuronal aspect of aging-related disorders and neurodegeneration.

5. EGFR Regulates Aging-Related Metabolic Activity

Aging is considered to be one of the most complex physiological processes known. Despite its enormous molecular complexity there appear to be several molecular mechanisms in which the aging process can be considered in a generic sense, for example, disruption of hormonal axes, accumulated oxidative stress, increasing nucleic acid instability, and a reduction of metabolic efficiency. Even among these common aging factors, metabolism for one appears to be vital for all of the physiological aspects of aging. It is not surprising therefore that many of the effective antiaging strategies intersect with this important factor [109, 124–130]. Therefore, if metabolic function is an overarching factor in the aging process, the question arises as to how the multifunctional EGFR might affect this profoundly important process.

Historically, however, the EGFR has been more commonly associated with cancer biology as opposed to neuronal function, metabolism, or aging. Given that the EGFR is one of the most widely expressed growth factor receptors, it is implicated in playing an important role in the proliferation of a wide variety of cell types in addition to the previously studied cancerous cells [131]. For example, the EGFR is amplified and overexpressed in tumors of epithelial [131] and glial origins [132] as well as other numerous neurometabolic-related tumors observed in humans. Viruses often associated with cancers, such as the hepatitis B virus, which is associated with hepatocellular carcinoma, lead to upregulation of EGFR signaling [133, 134]. Recent research however has started to reveal a more widespread contribution of the EGFR to complex physiological processes such as energy metabolism. In gastric cell lines, transactivation

of the EGFR intimately involves leptin-signaling. Leptin is a cytokine hormone secreted by adipocytes that regulates body weight by decreasing food intake and energy expenditure [135]. EGFR is also constitutively expressed in epithelium and airway smooth muscle (ASM) [136] as well as in hepatocytes [137]. The liver represents one of the most important organs with respect to the global somatic control of both energy expenditure and nutrient storage. Interestingly, it has been reported that the EGFR demonstrate a 2-fold higher expression in adult rat males compared to females and this phenomenon may be influenced by secretory rhythm of growth hormone (GH) in the pituitary [138].

Perinatal deletion of EGFR in hepatocytes results in decreased body weight, whereas deletion in the adult liver does not appear to affect body mass. Although liver function was not affected, after partial hepatectomy, mice lacking hepatic EGFR exhibit increased mortality and elevated serum transaminases, indicative of liver damage. EGFR has also been shown to be a critical regulator of hepatocyte proliferation in the initial phases of liver regeneration [139]. Several studies have investigated various regulatory factors that contribute to the affinity of EGF to EGFR in rat hepatocytes, for example, thyroid hormones [140], age [141], sex [141], fasting state [142], liver regeneration [143], and an imposed experimental diabetic state [144–146]. In accordance with this, a decreased hepatic EGFR expression has been reported in diabetic mice [147, 148]. Given the aforementioned molecular connectivity between the EGFR and the insulin/IGF-1/GH signaling systems it is logical that insulin may be one of the most important factors that regulate EGFR gene expression in the liver [147]. EGF and EGFR have been shown to be involved in an antiapoptotic effect in mouse hepatocytes [149] while EGF decreases the glucose transporter 2 expression level in chicken hepatocytes via PKC-MAPK [150]. Insulin deficiency causes a decrease in EGFR and its mRNA gene expression: therapeutic introduction of exogenous insulin restored the EGFR expression to control levels [147]. In addition to the insulinotropic regulation of hepatic EGFR function, the EGFR can be tyrosine-phosphorylated in the liver in a time- and dose-dependent manner in response to GH [151].

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is a cell adhesion protein and recently identified a substrate of the EGFR [152]. Once phosphorylated by EGFR activation, CEACAM1 appears to mediate increases in insulin sensitivity and can decrease insulin-dependent mitogenesis *in vivo*. An attenuated response to insulin stimulation is causally related to obesity, type 2 diabetes, and metabolic syndrome, and improved whole-body insulin sensitivity is important in the treatment of these metabolic disorders [153]. CEACAM1 therefore appears to play a central role in connecting EGFR activity with the control of central obesity/insulin resistance. The connection between EGFR activity and obesity is further reinforced by the observation that obesity is strongly associated with an increased mortality rate in cancer patients [154, 155]. The mechanism(s) linking altered metabolic pathways to mitogenic activity may involve EGFR activation by fatty acids independently of the cognate growth factor ligands of

EGFR [156, 157]. Free fatty acids (FFAs) are upregulated in fasting plasma of obese individuals [158]. Rather than being considered a passive organ adipose tissue is now appreciated as a dynamic endocrine regulatory organ. In addition to releasing FFAs [159] HB-EGF can be released from adipose tissue in obese individuals into the portal circulation [160]. Both FFAs and HB-EGF possess the capacity to engender EGFR activation in epithelial cells [152].

As we have discussed, in addition to its strong role in hepatic function, the EGFR also appears to coordinate multiple aspects of the glucose metabolic system. EGFR and the EGFR ligands, especially TGF- α , is functionally expressed in the developing pancreas [161]. EGFR expression is essential for normal pancreatic development and important in postnatal β cell growth [162]. Furthermore, EGFR signaling is essential for pancreatic islet β cell mass expansion during a high-fat diet and pregnancy. In these scenarios cellular replication appears to be the primary mechanism for the compensatory β cell mass expansion [163]. EGFR signaling however was not crucial to increase β cell proliferation after pancreatic duct ligation, which serves as a model for islet neogenesis. In EGFR-deficient mice, the development of β cells occurs at a later stage and the nascent β cells have a definite migratory defect [162]. EGFR ligands, EGF, HB-EGF, and betacellulin have all been implicated in β cell replication, differentiation and lineage determination of developing islet cells [164–167]. In addition to direct ligand-induced activation of the pancreatic EGFR, local GPCR-mediated transactivation of the receptor also appears to be potentially important in the EGFR-mediated regulation of glucose metabolism [168]. Glucagon-like peptide 1 (GLP-1) is secreted by intestinal L-cells in response to fat meals and carbohydrates [169–171]. In addition to its direct roles in the alimentary canal, for example, regulation of gastric emptying, GLP-1 also can functionally regulate β cell function in the pancreas, thereby providing another mechanism to control energy metabolism. The GLP-1 peptide activates its cognate Class B-type GLP-1 receptor to induce a c-Src-dependent EGFR transactivation process. This signaling system involves a proteolytic processing of membrane-anchored betacellulin, or other EGF-like ligands, that results in a strong PI3-kinase activation resulting in an effective increase in β -cell proliferation [172].

A common pathological aspect of metabolic aging is a slow inevitable generation of global insulin resistance [13]. Insulin resistance and the associated metabolic syndrome lead to a reduction in the ability of multiple cell types, and especially neurons, to uptake glucose, regulate calcium homeostasis and respond positively to trophic and neurotrophic stimuli [173]. In addition to generating a global disruption of neuronal energy balance, protracted insulin resistance leads to a loss of vascular smooth muscle cell (VSMC) functional regulation and promotion of VSMC migration [174]. The latter is crucial in atherosclerotic development and wound healing. Insulin has also been implicated in epidermal wound healing through EGFR signaling [174]. Insulin can induce transactivation of EGFR by ADAM-mediated HB-EGF-dependent process in VSMCs [174]. In quiescent fibroblasts, the mitogenic effect of EGF requires

activation of the IGF-1R by circulating ligands including IGF-I, IGF-II, or insulin [175]. As alterations in VSMC tone and function are likely to affect systemic hypertension, it is interesting to note that elevated plasma glucose can also result in the transactivation of the EGFR in renal disorders [176].

6. EGFR and TrkB Signaling: Potential Mediators of the Neurometabolic Interface

Considerable scientific progress has been made in studying EGFR since Cohen and Carpenter's publication (1975) that EGF induces precocious eyelid opening in neonatal mice [177]. The EGFR demonstrates an unprecedented number of functional interactions with other receptors, ligands, and molecules other than its own cognate ligands. Understanding these interactions is crucial to understanding the many different levels at which it is contributing to neurometabolic processes. However, the placing of the EGFR in such a central and important role in aging is overly simplistic. There are of course likely to be other factors that also possess a multidimensional impact upon neurometabolic function. For example, the interaction of the EGFR with the metal ion complexes leads to the resultant generation of downstream signaling phenotypes which are more characteristic of that engendered by the activation of the neurotrophic TrkB signaling system. The activity of the neurotrophic receptor family is already appreciated to be incredibly important to the presence and generation of AD pathology [178]. Trk receptors are necessary for the survival, differentiation and maturation of the developing brain [179]. Multiple lines of evidence have demonstrated a reduction of brain-derived neurotrophic factor (BDNF), the cognate ligand for the TrkB receptor, in the brain of AD patients [180–183]. Furthermore, TrkB has been reported to be important for LTP, a process considered to underpin memory function, in hippocampal CA1 neurons [184, 185]. Both the TrkB and EGFR signaling systems could potentially form a functional link that generates a combined regulatory system deeply associated with the majority of signaling systems controlling the aging process. It is interesting to note that both the TrkB and EGFR receptor systems can be directly linked through GPCR "transactivation" signaling mechanisms. Therefore it is likely that a "higher-order" synergy between TrkB and the EGFR could account for a significant component of the neurometabolic aging phenotype. Also through their common GPCR interaction in this "super-system" could be easily targeted in a therapeutic manner as currently over half of the effective pharmacopeia is designed to employ GPCR signaling systems. Such potential agents targeting this EGFR-TrkB network may be able to exert tremendously powerful therapeutic effects in aging and neurodegenerative paradigms.

7. Conclusion

In this paper we have attempted to uncover the context of neuronal and metabolic interaction at the molecular level during the aging process. Enormously complex systems

such as aging entail functional interaction of a myriad of physiological and molecular signaling systems. To provide a workable molecular framework on which to regulate and control such an enormous system it is likely that the existence of factors that can bridge, and adapt to, a multiplicity of signals, receptors, cell types, and tissues is crucial to the orchestration of the overall network. It is at this level that individual functional entities, such as the EGFR receptor system, may come to prominence. In its ability to interact and regulate multiple neuronal and metabolic functions across lifespan, the EGFR may indeed be one of the most important molecules whose enhanced study could yield future insights of tremendous importance for gerontology. In our paper we have drawn together evidence to aid the appreciation of the truly multidimensional role of EGFR at the systemic level in neurometabolic processes and in the neurodegenerative trajectories seen in the aging process.

Abbreviations

AD:	Alzheimer's disease
ADAM:	A disintegrin and metalloproteinase
Akt:	Ak-thymoma8
APP:	Amyloid- β precursor protein
AR:	Amphiregulin
ASM:	Airway smooth muscle
BDNF:	Brain-derived neurotrophic factor
BMPs:	Bone morphogenetic proteins
CA1:	Cornu Ammonis 1
CaM kinase II:	Ca ²⁺ /calmodulin-dependent protein kinase
c-Cbl:	Casitas B-lineage lymphoma protooncogene
c-fos:	Fbj osteosarcoma oncogene
c-Src:	Rous sarcoma oncogene
CNS:	Central nervous system
CQ:	Clioquinol
Cu:	Copper
DG:	Dentate gyrus
EGF:	Epidermal growth factor
EGFR:	Epidermal growth factor receptor
Eps15:	Epidermal growth factor receptor phosphorylation substrate-15
ER:	Epiregulin
ERK:	Extracellular-signal-regulated kinases
FFAs:	Free fatty acids
GH:	Growth hormone
GLP-1:	Glucagon-like peptide 1
GLP-1R:	Glucagon-like peptide 1 receptor
GPCR:	G-protein coupled receptor
Grb2:	Growth factor receptor-binding protein 2
HB-EGF:	Heparin-binding epidermal growth factor
IGF-1:	Insulin growth factor-1
IGF-1R:	Insulin growth factor-1 receptor
IRS-1:	Insulin receptor substrate-1
LHRH:	luteinizing hormone-releasing hormone
LIF:	Leukemia inhibitory factor
LPA:	Lysophosphatidic acid
LTP:	Long-term potentiation

MAPK:	Mitogen-activated protein kinase
MMPs:	Matrix metalloproteinases
NS/NPCs:	Neural stem and progenitor cells
PBMCs:	Peripheral blood mononuclear cells
PGE2:	Prostaglandin E2
PI3K:	Phosphoinositide 3 kinase
PKC:	Protein kinase C
PLC- γ :	Phospholipase C gamma
PTB:	Protein tyrosine binding domain
p-Tyr:	Phosphorylated tyrosine
Ras:	Rat sarcoma viral oncogene
RPTP- κ :	Receptor protein tyrosine phosphatase kappa
ROS:	Reactive oxygen species
RTK:	Receptor tyrosine kinases
Ser:	Serine
SH2:	Src homology 2 domain
Shc:	Src homology construct
Src:	Rous sarcoma protein
STAT3:	Signal transducer and activator of transcription 3
SVZ:	Subventricular zone
TACE:	Tumor necrosis factor alpha-converting enzyme
TGF- α :	Transforming growth factor alpha
TLR:	Toll-like receptors
TM:	Transmembrane
TNF- α :	Tumor necrosis factor alpha
Trk:	Tropomyosin receptor kinase
Tyr:	Tyrosine
UV:	Ultraviolet
VSMC:	Vascular smooth muscle cell.

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Research Article

Primary Hyperparathyroidism in Older People: Surgical Treatment with Minimally Invasive Approaches and Outcome

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Introduction. Elderly patients with primary hyperparathyroidism (pHPT) are often not referred to surgery because of their associated comorbidities that may increase surgical risk. The aim of the study was to review indications and results of minimally invasive approach parathyroidectomy in elderly patients to evaluate its impact on outcome. *Materials and Methods.* All patients of 70 years of age or older undergoing minimally approach parathyroidectomy at our Department from May 2005 to May 2011 were reviewed. Data collected included patients demographic information, biochemical pathology, time elapsed from pHPT diagnosis to surgical intervention, operative findings, complications, and results of postoperative biochemical studies. *Results and Discussion.* 37 patients were analysed. The average length of stay was 2.8 days. 11 patients were discharged within 24 hours after their operation. Morbidity included 6 transient symptomatic postoperative hypocalcemia while one patient developed a transient laryngeal nerve palsy. Time elapsed from pHPT diagnosis to first surgical visit evidences that the elderly patients were referred after their disease had progressed. *Conclusions.* Our data show that minimally invasive approach to parathyroid surgery seems to be safe and curative also in elderly patients with few associated risks because of combination of modern preoperative imaging, advances in surgical technique, and advances in anesthesia care.

1. Introduction

Primary hyperparathyroidism (pHPT) is one of the most common endocrine diseases with prevalence rates of about 3 per 1,000 in the general population and up to 1 per 100 in the elderly. The chances of developing pHPT increase with age, and patients are most often diagnosed in the sixth and seventh decades of life [1].

Changes in endocrine systems, including loss of skeletal mass, are common or certain in older persons. Skeletal mass increases in most individuals until about the age of 20. It remains stable until about the age of 35 and thereafter declines at a relatively steady rate throughout the remainder of life. Decreased skeletal mass associated with increasing age is the result of a series of changes associated with aging: calcium absorption/transport in the intestinal mucosa and mineral metabolism [2], vitamin-D-dependent calcium absorption, and calcium intake generally decreasing with age [3]. As renal function decreases with age, parathyroid

hormone (PTH) increases with augmented osteoclastic and osteoblastic activity [4–7]. Although, after the introduction of serum calcium determination in the routine biochemical screening in the early 1970s, most patients referred to surgery are asymptomatic, clinical manifestations of pHPT, including disabling fractures, are frequent in the elderly [8].

Elderly patients with pHPT are often not referred for surgical intervention because of their associated comorbidities that may increase surgical risk.

For the reason that pHPT is more common in the elderly [1] and because minimally invasive techniques in parathyroid surgery have recently been demonstrated safe with improved perioperative outcome [9–14], the aim of our study was to review indications and results of minimally invasive parathyroidectomy in patients 70 years of age and older (elderly) to evaluate the safety and efficacy of outpatient minimally invasive parathyroidectomy (MIP) and minimally invasive video-assisted parathyroidectomy (MIVAP).

2. Materials and Methods

A review was conducted of a prospectively collected database of all patients undergoing parathyroidectomy at the Department of General Surgery of University of Trieste. During the last 5 years, from May 2005 to May 2011, 83 patients with pHPT underwent parathyroidectomy. 37 (44.58%) patients of 70 years of age or older were reviewed. MIVAP by anterior approach, technique previously described [13, 15], was proposed for patients with sporadic pHPT due to a single gland disease, an adenoma smaller than 35 mm as demonstrated by preoperative imaging, unequivocally preoperative localization by ultrasonography and ^{99m}Tc-SestaMIBI scanning, no associated giant goiter, no suspected carcinoma of the thyroid, no secondary or recurrent hyperparathyroidism, no previous neck surgery, and no previous radiation to the neck. We used the operative technique first described by Miccoli et al. in 1997 and 1998 [16, 17], without carbon dioxide insufflation. The procedure, in which four surgeons are involved (first operator, one surgeon assisting the first operator, one surgeon holding retractors, and one surgeon holding the endoscope), was performed through a single 20 mm skin incision in the central neck, 1-2 cm above the sternal notch. The patient, under general anaesthesia, is placed in a supine position; the neck is in slight extension. Dissection was performed under endoscopic vision, using small conventional retractors and needlescopic (2 mm) reusable instruments. Video assistance was obtained using a 30-degree 5 mm endoscope. The thyroid lobe was retracted medially and the adenoma was extracted after clipping its pedicle.

MIP, defined as open “directed” parathyroidectomy (unilateral targeted cervical exploration) with a minimally invasive approach (smaller cervical incision and less cervical dissection than traditional open technique necessitating bilateral cervical exploration), was proposed for patients with sporadic pHPT due to a single gland disease, preoperative localization of one enlarged parathyroid gland on ultrasonography or on ^{99m}Tc-SestaMIBI scanning, no associated suspected thyroid malignancies, and no secondary or recurrent hyperparathyroidism.

MIP was proposed when preoperative ultrasonography did not localize certainly the adenoma or when there were benign thyroid nodules associated with pHPT.

Technically, MIP is the open focused procedure that is performed without the videoscope and requires greater neck extension, a 3 to 5 cm incision, and larger subplatysmal flaps than MIVAP. In all other aspects, the procedure is practically identical.

Intraoperatively, a quick parathyroid assay was used to measure intact parathyroid hormone levels during the last 25 surgical procedures (PTHIO): before surgery and then 5, 10, and 15 minutes after excision of the adenoma, peripheral blood was drawn.

Intact PTH assay, both in routine and intraoperative mode, was performed on samples collected into potassium EDTA anticoagulant tubes according to the routine and intraoperative procedure of the Access Immunoassay System Intact PTH, respectively, a paramagnetic particles,

chemiluminescent immunoassay for the quantitative determination of PTH levels in human serum and plasma on Access2 Beckman Coulter (Fullerton).

The calibration, valid up to 28 days, was performed for routine and intraoperative modes using separate calibration cards and two control levels, low and high, were double assayed before and after surgical operation. The assay imprecision was evaluated by testing 2 levels of controls generating a total of 20 assay, 2 replicate per assay, over 10 days [18].

The analytical range for PTH and PTHIO assay was 1–3500 pg/mL, and 6–3500 pg/mL respectively; the reference interval for PTH values was 11–73 pg/mL.

A decrease in iPTH levels of more than 50% in the 5-minute postexcision sample below the preincision value, as suggested by Irvin et al. [19, 20], was the criterion used to indicate that the offending gland has been excised and the remaining parathyroids glands were not hyperfunctioning.

In summary, the surgical procedures were considered successful when more than a 50% decrease in preexcision PTH levels was observed after 5 minutes.

Preoperative informed consent was obtained from all patients [15].

pHPT was biochemically confirmed before surgery, and patients were selected for parathyroidectomy based on symptoms and according to guidelines for surgical intervention in asymptomatic pHPT. Preoperative planning included preoperative calcemia (normal range: 8.50–10.50 mg/dL), and PTH levels (normal range: 11–73 pg/mL). All patients underwent preoperative investigations of vocal cord function.

Data collected included patients’ demographic information, biochemical pathologic, time elapsed from pHPT diagnosis to surgical intervention, PTHIO measures operative findings, complications, and results of postoperative biochemical studies.

2.1. Statistical Analysis. All patients of 70 years of age or older, undergoing parathyroidectomy at our department from May 2005 to May 2011, were included in a database. In an Excel spreadsheet were entered data of patients, surgical procedures, length of surgical procedures, length of stay, biochemical analysis, cytology, histology, PTHIO measures, complications, and results of postoperative biochemical studies.

Statistical analysis was performed using the “R 2.13.1” software (<http://www.rproject.org/>).

Continuous data were expressed as the mean or median \pm standard deviation, as appropriate, while discrete data as a finite value or percentage.

The population’s ages were forced with two box plots display (Figures 1 and 2) to show graphically the distribution of elderly population. Figure 1 shows all elderly patients in relation to age while Figure 2 represents the differences between elderly males and elderly females regarding age.

The Fisher exact test was employed to correlate the complications between the two techniques.

A *P* value less than 0.05 was considered statistically significant.

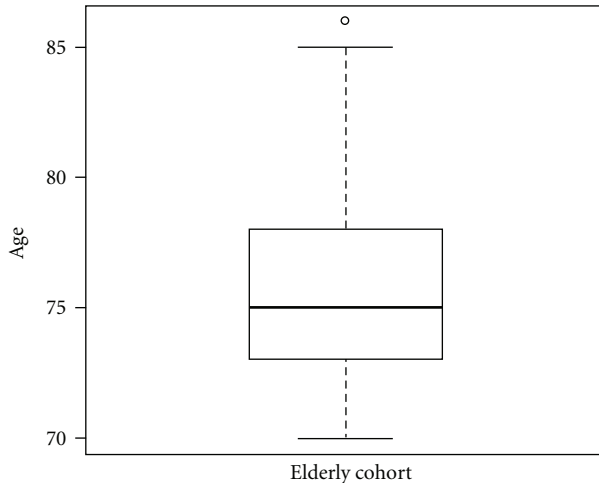


FIGURE 1: Box plot regarding age. It represents the distribution by age of pHPT in our elderly cohort made up of 37 patients during the last 5 years of activity of the Department of General Surgery of University of Trieste. Elderly mean age at time of operation was 76.08 years (range: 70–86 years). The median age was 75 years and corresponds to the dark thickened band near the middle in the box plot, drawn vertically. The box in the figure shows the lower and upper quartiles of age (range 73–78 years) while whiskers show values below the 25th (from 70 to 72 years) and above the 75th (from 79 to 85 years). The upper dot not included between the whiskers means a patient 86 years old at time of operation.

3. Results and Discussion

During the last 5 years, from May 2005 to May 2011, 37 patients, 28 women and 9 men, of 70 years of age or older were analysed. Mean age at time of operation was 76.08 ± 4.2 (range: 70–86 years), and median was 75 (Figure 1). For females, mean age was 75.68 ± 4.5 (range: 70–86 years), and median 74; for males, respectively, 77.33 ± 2.6 (range: 74–82 years) and 78 ± 2.6 (Figure 2).

Mean time elapsed from pHPT diagnosis to surgical intervention was 9 months ranging from 2 to 36 months.

Mean preoperative serum calcium level was 11.43 mg/dL (range: 10.3–15 mg/dL). Among the 37 elderly patients who had parathyroidectomy, 11 (29.73%) had concomitant thyroid surgery. The mean overall PTHIO drop was a drop of 18.38% in T1 and 59.91% in T2.

Totally, 29 MIP and 8 MIVAP were performed.

Mean operative time was 66 minutes (range: 32–100 minutes) for MIP and 96 for MIVAP (range: 40–148), respectively. In all patients final histology showed benign disease. Surgical cure of pHPT was achieved in all patients with serum calcium levels normalization.

The average length of stay was 2.8 days (range: 1–13 days). 11 patients (29.73%) were discharged within 24 hours after their operation.

Overall morbidity was 18.91% (7 patients), and precisely postoperative complications included 6 (16.21%) transient symptomatic postoperative hypocalcemia (complete recovery after 13 days) while one patient developed transient laryngeal nerve palsy (2.70%).

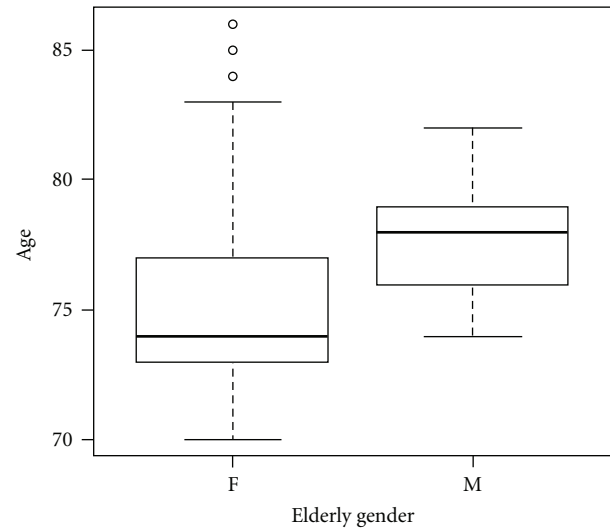


FIGURE 2: Box plot regarding age and sex. The figure shows the distribution by age, represented vertically, and gender, horizontally, of pHPT in our elderly cohort. The box plot highlights that, over 5 years of activity of our Department, there was a major incidence of pHPT in females than males (28 versus 9) and that the age of onset of disease was lower in elderly females than elderly males. In fact, for females, the median age was 74 years and lower and upper quartiles of age ranged from 73 to 77 years; 3 outlier patients 84, 85, and 86 years old at time of operation. On the other side, for males: the median age was 78 years and lower and upper quartiles of age ranged from 76 to 79 years.

No definitive laryngeal nerve palsies, no definitive hypocalcemia (lasting more than 6 months after surgery), no persistent pHPT, and no recurrent pHPT were observed.

Postoperative complications in MIVAP group included 1 (12.5%) transient hypocalcemia, whereas, in the MIP group, postoperative complications included 5 (17.24%) transient hypocalcemia ($P = 1.458$) and 1 (3.44%) temporary laryngeal nerve palsy (complete recovery after 1 month) ($P = 0.8947$).

At a mean follow-up of 31 months ranging from 6 to 66 months, all patients are normocalcemic.

Primary hyperparathyroidism is a common endocrine disorder in the elderly. After the introduction of routine serum calcium measurements in the 1970s, facilitating the diagnosis of asymptomatic pHPT, epidemiological studies from the United States and Europe reported a higher prevalence than previously presumed [1, 21].

In a literature review, in accordance with an age-dependent increase in pHPT, the population-adjusted incidence of parathyroidectomy resulted in being higher in persons aged ≥ 50 years. The peak of pHPT incidence was observed in patients aged 70–74 years (12.7/100,000), with a decline over 75 [21, 22].

Parathormone secretion tends to increase slightly with age. The relation can be attributed to decreased calcium and vitamin D intake (and possibly decreased sun exposure) and to kidney failure as causes a reduction of 1,25hydroxilation of vitamin D.

Bone mass, influenced by the effect of PTH, declines gradually with age; the decline accelerates after menopause in women and continues indefinitely. This loss of bone contributes to an increased risk of fracture and increased probability of falls.

Since the introduction of routine calcium screening and multichannel biochemical testing, the majority of pHPT patients are diagnosed at an early stage and pHPT patients are commonly referred to as “asymptomatic.” Guidelines for managing asymptomatic pHPT have been outlined using objective indications for surgical intervention [23]. Surgical removal of abnormal parathyroid tissue is the only curative treatment for pHPT.

Despite a continuing increase in pHPT hospitalisation rates with older age, elderly patients are often not referred for surgical intervention [24]. Several factors could affect the decision of surgery: the age, the number of comorbidities, and the remaining life expectancy.

Time elapsed from pHPT diagnosis to first surgical visit evidences that the elderly patients in our series were referred after their disease had progressed, with advanced bone disease, and very high PTH and calcium serum levels.

Although encouraging outcomes of surgical treatment in elderly patients with pHPT have been reported [9–14] there may exist a reluctance to offer the surgical intervention in particular from endocrinologists; moreover, elderly patients often fear parathyroidectomy. In fact, the reason of this delay is not only ascribed to physician or surgeon. It is necessary to take in account the psychological aspect of elderly patients who often admit their panic versus surgical intervention and then their refusal. Elderly patients as a result are often not allowed to improve in quality of life, bone health, neuromuscular function, psychiatric symptoms, and the decrease in morbidity and mortality that are associated with cure of pHPT [25–27].

Although medical therapy exists for the decreased bone mass caused by pHPT, this therapy based on Calcium Sensor Mimetics is often not tolerated while surgical therapy is definitive and offer better results.

In the past surgeons and physicians have been less inclined to operate on or to refer to surgery elderly pHPT patients because of the risks associated with standard cervical exploration, general anesthesia, endotracheal intubation, long hospital stay, and postoperative complications.

But it is necessary to consider that although, historically, pHPT has been treated by standard cervical bilateral (four glands) exploration, single adenomas are responsible for pHPT in >80% of patients and resection of the gland involved is curative [28, 29].

In recent years, improved preoperative localization techniques and the availability of intraoperative parathyroid hormone monitoring have opened the way for minimally invasive procedures, and several procedures have been described [16, 17, 30–32]. Previous studies have shown that parathyroidectomy today can be performed as safely in elderly patients as in their younger counterparts [14, 33] with low complication rate also in elderly patients and with discharge after an overnight stay.

In our series all patients underwent general anesthesia, but in the literature are described also minimally invasive parathyroidectomy for primary hyperparathyroidism, under light sedation, or using locoregional cervical block anesthesia [34–36]. These new advances in anesthesia care reflect the possibility to operate on also patients who are not eligible for general anesthesia, for example, for serious associated cardiovascular comorbidities.

In this study, we present our results of elderly patients who underwent parathyroidectomy by minimally invasive approach. About 30% of patients were discharged within 24 hours after their operation. Mean hospital stay was about 2–3 days, data similar with other results reported for younger patients [10, 11, 14]. Also the morbidity in this series is not dissimilar to other reports [14, 16]. Anyway, it is indispensable to consider, as reported by Thomas et al. [37], that elderly patients sustain more morbidity following parathyroidectomy related to their comorbidities such hypertension and coronary and pulmonary diseases than those related to surgical procedure. Moreover, older individuals often live alone and early discharge is very difficult. In our series, one patient had a very long hospital stay (13 days). There was a 76-year-old woman who underwent minimally invasive parathyroidectomy and total thyroidectomy associated, in which symptomatic postoperative hypoparathyroidism occurred and, in spite of substitutive therapy, serum calcium levels resulted at all controls in being lower than normal values. Furthermore, the patient was not autosufficient with various disabilities and asked to remain in hospital until complete normalization of serum calcium levels.

4. Conclusions

Our data show that minimally invasive approach to parathyroid surgery, either video assisted or with only open smaller neck incision and unilateral cervical exploration, seems to be safe and curative also in elderly patients. Furthermore, risks associated to this kind of surgery are nowadays considerably lower because of the combination of modern preoperative imaging, advances in surgical techniques and advances in anesthesia care. In view of this we recommend early surgical referral for pHPT in all patients regardless of age. Further research, with a greater number of patients is necessary to identify a precise benefit-risk assessment.

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Review Article

Partial Androgen Deficiency, Depression, and Testosterone Supplementation in Aging Men

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The aim of this review was to summarize current knowledge on the correlation between depressive symptoms with a syndrome called partial androgen deficiency of the aging male (PADAM) and on the potential benefits of testosterone (T) treatment on mood. Despite the causative nature of the relationship between low T levels and depression is uncertain, many hypogonadal men suffer from depression and vice versa several depressed patients are affected by hypogonadism. Supplementation with testosterone failed to show sound evidence of effectiveness in the treatment of depression. Nevertheless, testosterone supplementation has proved to be effective on some domains significant for the quality of life of aged patients with PADAM (sexual function and cognitive functions, muscular strengths).

1. Introduction

Testosterone deficiency or hypotestosteronemia is a widely recognized hormonal alteration associated with male aging [1–3]. Its prevalence may be as high as 30% in men aged 40–79 years [4, 5], and in up to 12% the hypotestosteronemia it can be associated with clinical symptoms [5]. Nevertheless, different levels of testosterone (T) could be associated with the presence of specific clinical symptoms [6, 7].

The joint consensus of International Society of Andrology, the International Society for the Study of the Aging Male (ISSAM) and the European Association of Urology prepared a set of recommendations specifically on the investigation, treatment, and monitoring of late-onset hypogonadism in males [8, 9]. Laboratory diagnosis of hypogonadism is based on the measurement of serum total testosterone (TT). Although there is no uniformly accepted threshold level for T in older men, TT levels above 350 ng/dL are considered normal and do not require substitution therapy, while TT

levels below 230 ng/dL usually benefit from testosterone treatment. When TT level is between 230 and 350 ng/dL, it may be useful to calculate free testosterone (FT), particularly in obese men. FT level below 65 pg/mL suggests that testosterone treatment is needed [10, 11].

In 2010, the Endocrine Society published clinical practice guidelines for testosterone therapy in adult men with androgen deficiency syndrome [12]. The members of the working group agreed that because the normative ranges for TT and FT in healthy young men vary among laboratories and assays (lower TT limits: 280–300 ng/dL; lower FT limits: 5–9 pg/mL) [13], clinicians should use the lower limit of normal range for healthy young men established in their laboratory. Members of the working group disagreed on T concentrations below which testosterone supplementation should be offered to older men with symptomatic hypogonadism. Some members of the working group recommended T supplementation in older men with TT level below 300 ng/dL, because this is the threshold at which older men have

symptoms that might be attributable to low testosterone; others recommended T supplementation only in those with TT level below 200 ng/dL, because higher pretreatment T values are associated with lower beneficial effects of T therapy.

Age-related serum testosterone decline is caused by different simultaneous mechanisms, such as primary structural gonadal impairment, age-related degenerative modifications of the pituitary gland, deficits of the neurohypothalamic system, and primary peripheral metabolic abnormalities such as the age-associated increase in the concentration of serum sex hormone binding globulin (SHBG), with a consequent decrease in FT [3].

It is controversial whether aging is to be considered as the only variable linked to age-related T decline [14, 15]: several factors do seem to interfere in different ways with T metabolism, like genetic factors [16], chronic diseases [17–19], chronic medications [20], obesity [7, 21, 22], and lifestyle factors [23, 24].

Despite the fact that many men with low testosterone levels are asymptomatic [25], many others have a partial, gradual, and variable decline in T associated with various clinical symptoms, described as a syndrome called partial androgen deficiency of the aging male (PADAM) [26]. PADAM is characterized by sexual, somatic, and behavioral symptoms, with insidious onset and slow progression [27]: diminished sexual desire and erectile quality, particularly nocturnal erections [28, 29]; decrease in lean body mass, with associated diminution in muscle volume and strength; increase in visceral fat [30–32], decrease in bone mineral density, resulting in osteoporosis [33]; reduction in body hair and skin alterations [34]; weakness, fatigue, depression, lack of motivation and energy, lower psychological vitality, anxiety, irritability, insomnia, decreased work and sport performances; difficulty in concentrating, memory impairment, and low dominance [35–41].

In the Endocrine Society Guidelines symptoms are separated into two groups, more specific symptoms and signs of hypogonadism (incomplete or delayed sexual development, sexual disorders, breast discomfort, gynecomastia, loss of body [axillary and pubic] hair, reduced shaving, very small or shrinking testes, inability to father children, low or zero sperm count, height loss, low trauma fracture, low bone mineral density, hot flushes, and sweats) and less specific symptoms (decreased energy, motivation, initiative, self-confidence, feeling sad or blue, depressed mood, dysthymia, poor concentration and memory, sleep disturbance, increased sleepiness, mild anemia, reduced muscle bulk and strength, increased body fat, body mass index, and diminished physical or work performance) [12]. Serum T concentration has to be measured in patients with the more specific symptoms of hypogonadism and considered in those who report the less specific symptoms. The diagnosis of hypogonadism is possible when serum T level is below lower limits, and reversible illness, drugs, and nutritional deficiency have been excluded.

PADAM as a clinical entity is still controversial, because it is very difficult to distinguish to what extent the symptoms attributed to PADAM are due to the natural and unavoidable

consequences of aging and how much to androgen deficiency [37, 42, 43].

Behavioral aspects of PADAM may overlap with signs of depression. For example, McIntyre et al. [44] considered that reduction in physiologically active bioavailable testosterone (BT) concentration is a vulnerability factor for depressive symptoms in middle-aged depressed men. The authors assessed and compared TT and BT levels in two groups of middle-aged men (40–65 years), untreated subjects meeting DSM-IV-TR (Diagnostic and Statistical Manual of Mental Disorders—4th edition, text revised) [45] criteria for a major depressive episode ($N = 44$), and a matched nondepressed control group ($N = 50$). Depressed men had lower mean BT levels and TT levels than the control group. Biochemical hypogonadism (i.e., BT level ≤ 70 ng/dL or TT level ≤ 350 ng/dL) was also more prevalent in depressed men than in nondepressed controls (34% versus 6%; 61% versus 14%, resp.).

Thus, the aim of this review was to summarize current knowledge on depressive symptoms correlated with PADAM and on the potential benefits of T treatment on mood.

2. Methods

In order to provide a critical review of the association of PADAM and depression in older males, we performed a PubMed search to identify all papers published in English peer-reviewed journals between 1980 and 2012. The search string was androgen deficiency OR testosterone deficiency OR hypogonadism OR testosterone treatment OR testosterone supplementation AND depress*.

We limited the search to articles reporting data for male aged 45+ years old. All English full-text articles reporting original data about the main topic were included. The reference lists of the articles included in the review were also manually checked to retrieve other relevant studies.

3. Results

3.1. T Levels and Depression. Epidemiological and clinical studies of the connection between age-related low T levels and a reduced feeling of well-being, with unusual anxiety and irritability, nervousness, mood swings, and a depressive state, have produced mixed results [46–63] (see Tables 1 and 2).

Positive results were reported by Hintikka et al. [47] who examined associations between hypogonadism (laboratory diagnosis was based on FT level < 4.6 ng/dL), erectile dysfunction, sexual desire, and long-term and current depressive symptoms in a population-based sample of Finnish middle-aged men. The inclusion criteria for this study were based on self-reported adverse mental symptoms prevailing at baseline and at the 3-year followup. At 7 years from the baseline, men who reported long-term adverse mental symptoms had higher depression but lower FT levels than asymptomatic men. Furthermore, depression correlated negatively with FT ($\rho = -0.20$; $P < 0.05$) in the entire sample.

The Rancho Bernardo Study examined the association between T and depression in 856 community-dwelling older

TABLE 1: Naturalistic and cross-sectional studies in nonselected samples of patients.

Author, year	Type of study	Sample size	Age	Outcome measures	Results
Barrett-Connor et al. [64]	Cross-sectional population-based study	856 men	Range: 50–89 yrs	Association between BDI and BT or DHT	BDI scores were inversely associated with BT and DHT. BT were 17% lower for men with categorically defined depression than levels observed in all other men.
T'Sjoen et al. [105]	Naturalistic	236 male outpatients in 1997 and 192 in 2000	All patients were 70+ yrs. Range: 73.5–78.5 yrs in 1997; 76.0–81.0 yrs in 2000	Association between GDS and TT, FT, estradiol, DHEAS, AR gene CAG-repeat length	No relationship between the GDS and FT or TT in 1997. The GDS did not correlate with AR gene CAG-repeat length. The GDS of 1997 correlated significantly with (free) estradiol and with DHEAS. The analysis in 2000 confirmed a lack of association between GDS and androgen levels or AR gene CAG-repeat length. The association between GDS and (free) estradiol and DHEAS was not observed in the subgroup of men studied in 2000. Changes in FT and serum (free) estradiol levels between 1996 and 2000 were not related to GDS score in 2000.
Strasser et al. [55]	Naturalistic	48 patients with cancer who had received no major antineoplastic intervention for at least 2 weeks	Range: 20–79 yrs; Median: 59 yrs	Association between HAM-D and hypogonadism	64% of patients had hypogonadism, which was correlated with HAM-D score.
Spetz et al. [43]	Cross sectional	370 men	Range: 55 to 75 yrs; median: 62 yrs	Blood concentrations of T and BT	Men reporting deterioration in work performance had significantly lower T (16.6 nmol/L) and BT (6.9 nmol/L) than men without this problem (18.8 nmol/L and 7.9 nmol/L, resp.). Men reporting decreased strength and/or endurance had lower concentrations of BT than men without the same complaint (7.2 nmol/L and 8.0 nmol/L, resp.). Men suffering from hot flushes had lower T (15.1 nmol/L) and BT (6.58 nmol/L) compared with men who either had flushes but were not bothered by them or men without any flushes (17.6 nmol/L and 7.63 nmol/L).
Kratzlik et al. [54]	Naturalistic	669 manual workers	Range: 43–67 years	Association between BT, BMI and BDI scores	There was a U-shaped (quadratic) association between BT and BDI. Obese and underweight men with high BT had an increase BDI score. Men with low BT levels had an increased BDI score and eugonadal men with normal T levels have the lowest risk of depression.
Morsink et al. [53]	Prospective cohort study	2855 well-functioning elderly men (1406) and women (1449)	All aged aged 70–79 yrs; Mean: 73.8 yrs (men); 73.5 yrs (women)	Associations between TT, FT, DHEAS and CES-D scores	Significant inverse association between DHEAS and the CES-D for men and women. In men, there was a borderline significant inverse association between TT and depressive symptoms. For men, those in the lowest quartile of DHEAS and TT had significantly more depressive symptoms than those in the other quartiles.

TABLE 1: Continued.

Author, year	Type of study	Sample size	Age	Outcome measures	Results
Makhlouf et al. [52]	Naturalistic	157 men referred to an erectile dysfunction specialty clinic	Range: 21–85 yrs; Median = 53 yrs	Scores of 22 or higher on the CES-D (overt depression)	Hypogonadal men were 1.94 times more likely to have overt depression scores compared to eugonadal counterparts.
Fukai et al. [50]	Cross-sectional	108 men and 100 women	Range: 70–95 yrs; Mean: 82 ± 7 yrs (men); 81 ± 6 yrs (women)	Associations between GDS and TT, FT, DHEA, DHEAS, Estradiol	Linear regression model of hormone levels on functional scores unadjusted and adjusted for age, and age and body mass index indicated that in men the GDS was not associated with hormone levels.
Hintikka et al. [47]	Naturalistic	59 men with adverse mental symptoms (AMS) and 57 asymptomatic (AS) men from the Finnish National Population Register	Mean: 55.9 ± 8.6 yrs for AMS and 56.3 ± 10.4 yrs for AS	Hypogonadism (FT < 160 pmol/L)	AMS more often had hypogonadism; BDI score and HAM-D correlated negatively with FT in the entire sample.
Wu et al. [7]	Naturalistic	Random population sample of 3,369 men at eight European centers	Mean: 59.7 ± 0.3 yrs	Associations between TT and FT, and items from the SF-36 and the BDI	Sadness (SF-36), loss of energy (BDI), and fatigue were significantly associated with FT but not with TT. The probability of symptoms increased with decreased levels of T. The thresholds for TT were approximately 160 pmol/L for both sadness and fatigue.
Jankowska et al. [49]	Cross-sectional	Study population: 163 men with stable systolic chronic heart failure (CHF), and 316 healthy men living in the same area	Range: 35–80 yrs (controls) Mean: 60 ± 10 yrs (CHF)	Associations between BDI and TT or DHEAS	There were no differences in serum TT levels between the healthy men and the men with CHF when evaluated according to BDI score. Men with CHF had lower serum DHEAS as compared with healthy men in subsequent groups according to BDI score. Lower TT and DHEAS were associated with depressive symptoms in men with CHF.
Wong et al. [106]	Naturalistic	1147 community-dwelling elderly men, aged	65+ yrs	Associations between GDS, DHEA, TT, FT, estradiol, and SHBG	DHEA was significantly associated with GDS score. No association was seen between depressive symptoms and TT, FT, estradiol levels or SHBG levels.
Berglund et al. [56]	Naturalistic	3413 men participating in the fifth Tromsø study in 2001	Range: 60 ± 14 yrs	Associations between the Hopkins Symptom Checklist-10 (SCL-10) scores and T	Men with T levels in the lowest 10th percentile, had increased SCL-10 score compared to men with higher T levels. However, men with more pronounced symptoms indicating mental disorder did not have lower testosterone levels.
Halabi et al. [63]	Cross-sectional	104 men with COPD; 36 of whom had significant depressive symptoms (GDS ≥ 11)	All 55+ yrs. Mean: 66 ± 1 (depressed), 71 ± 1 (nondepressed);	Associations between GDS and hypogonadism	Prevalence of hypogonadism was greater in patients with severe depressive symptoms (GDS ≥ 19) than in those with mild depressive symptoms (GDS = 11–18) (62% versus 26%). After controlling for confounders, however, gonadal state was not associated with severe depressive symptoms.

T: testosterone; BT: bioavailable testosterone; FT: free testosterone; DHEA: dehydroepiandrosterone; DHEAS: dehydroepiandrosterone sulphate; SHBG: sex hormone-binding globulin; BMI: body mass index; HAM-D: Hamilton scale for depression; BDI: Beck depression inventory; CES-D: center for epidemiologic studies depression scale; GDS: geriatric depression scale.

TABLE 2: Naturalistic and cross-sectional studies in patients with depressive disorders.

Author, year	Type of study	Sample size	Age	Outcome measures	Results
Steiger et al. [61]	Pre-post analyses	12 male patients with MDD	Mean: 46.4 ± 11.3 yrs	Change in nocturnal secretion of T	Nocturnal secretion of T increased after remission of depression.
Booth et al. [71]	Naturalistic	4,393 men from the Vietnam Experience Study	Range: 30–48 yrs; Mean = 37 yrs	Associations between T and depression measured with the items from the Diagnostic Interview Schedule	T-squared has a significant relationship with depression, indicating a curvilinear relationship. Among men whose testosterone is below 590 ng/dL, each increase in the testosterone level is associated with less depression. Among men whose testosterone is above this value, each increase in the hormone is associated with greater depression.
Schweiger et al. [60]	Cross-sectional	15 male inpatients with moderate to severe MDD and 22 healthy comparisons	Range: 22–85 yrs; 48 ± 15 for MDD versus 53 ± 18 for comparisons	Differences in T between groups	Daytime T, nighttime T, and 24-hour mean T secretion were significantly lower in the MDD inpatients.
Seidman et al. [62]	Cross-sectional	32 elderly men with dysthymic disorder, 13 elderly men with MDD, and 175 nondepressed comparisons	Range: 60–82 yrs	Differences in TT	TT was lower in elderly men with dysthymic disorder than in men with MDD and men without depressive symptoms.
Shores et al. [67]	Naturalistic	278 men with consistently normal or low T levels (TT ≤ 200 ng/dL; or FT ≤ 0.9 ng/dL) at baseline and during a 2-year follow-up period	45+ yrs	Differences in prevalence of ICD-9-CM depressive illness	The hypogonadal men had an increased occurrence of diagnosed depressive illness (21.7% versus 7.1%).
McIntyre et al. [44]	Cross-sectional	44 depressed and 50 nondepressed men	Range: 40–65 yrs; depressed= 52.0 ± 7.1; controls= 50.8 ± 6.5	Differences in T levels	Depressed men had lower BT levels and TT levels than controls. Biochemical hypogonadism (i.e., BT level ≤ 2.4 nmol/L or TT level ≤ 12.14 nmol/L) was also more prevalent in depressed men than in non-depressed controls (34% versus 6% and 61% versus 14%, resp.).
Ponholzer et al. [51]	Naturalistic	247 men of a population-based study	Mean: 75.7 yrs	Differences in T levels and DHEAS	T levels were not associated to prevalence or incidence of depression or dementia.

T: testosterone; BT: bioavailable testosterone; FT: free testosterone; DHEA: dehydroepiandrosterone sulphate; SHBG: Sex hormone-binding globulin; BMI: body mass index; HAM-D: Hamilton scale for depression; BDI: Beck depression inventory; CES-D: center for epidemiologic studies depression scale; GDS: geriatric depression scale; MDD: major depressive disorder.

men aged 50 to 89 years (mean age 70.2 years) during a period of 4 years [64]. In that study, BT levels decreased with age and were significantly and inversely associated with Beck Depression Inventory (BDI) scores [65], indicating more depressive symptoms associated with lower BT levels. There was a graded stepwise decrease in BT, with a parallel increasing level of depressed mood; in addition, BT levels were 17% lower in men with categorically defined depression than in controls.

Lee et al. [66] investigated the PADAM in a community sample of 311 Chinese men (aged 40–80) attending a family medicine clinic in Hong Kong. A total of 87.8% of the sample was screened PADAM positive using the ADAM questionnaire. PADAM-positive individuals were found to have poorer quality of life, higher depression and anxiety, even after adjusting for age and number of current diseases.

In a historical cohort study, the Veterans Affairs Puget Sound Health Care System carried out on 278 men aged 45 years and older without previous depressive diagnosis, hypogonadal men with TT levels of 200 ng/dL or less (compared to eugonadal men) showed an approximate 4-fold increase in the risk of incident depression in the 2-year followup [67]. The risk of depression was inversely related to T levels, with statistically significant findings observed at T levels lower than 280 ng/dL.

In a sample of 32 subjects with dysthymic disorder (mean age 70.5, SD 5.8, range = 60–82 years), Seidman et al. [62] found that most elderly men with dysthymic disorder had TT levels in the hypogonadal range (i.e., ≤ 300 ng/dL). Furthermore, their TT levels were lower than in patients with MDD or healthy controls. The authors hypothesized that dysthymic disorder in elderly men may be related to HPG axis hypofunction. This association is believed to be the result of either chronic depression leading to HPG axis blunting, or to HPG axis hypofunctioning leading to low-grade depression [68].

Furthermore, there is some evidence that, compared with controls, T secretion is blunted among older men with severe major depressive disorder (MDD), it appears to normalize after major depressive episode remission [60, 61]. A significant increase in depression during the androgen deprivation treatment period, and a tendency to decline after chemical castration was discontinued has been observed in eugonadal men at risk for prostate cancer who are treated with androgen blockade therapy [69].

No association between depression and T level has been reported in the Massachusetts Male Aging Study, a cross-sectional, population-based multidisciplinary survey of 1,709 normally aging men (aged 39–70 years) [70]. Partially positive results were also reported more recently in the Tromso Study [56]. In this study, lower testosterone levels were associated with subthreshold symptoms of anxiety and depression. The Veterans' Experience Study [71], which investigated a sample of 4,393 veterans who served the U.S. military (mean age 37 years), found small but significant associations between depression and T Level ($r = 0.04$; $P < 0.01$). However, in this latter study, the authors pointed out that the relationship between T level and depression may actually be curvilinear [71].

The causative nature of the relationship between low T levels and depression is uncertain. For example, investigators of the Massachusetts Male Aging Study found a significant interaction between polymorphic CAG repeats sequence encoding a variable-length glutamine chain in the N-terminal transactivation domain of an androgen receptor genetic polymorphism protein, testosterone level, and depression [72]. The CAG repeat length appears to have modulatory effects on androgen action [73, 74], and the associations between depression and testosterone concentration may be mediated by different androgen sensitivity. The psychiatric effects of T may be also mediated through modulation of brain monoamine levels and, in particular, of the serotonergic function [75, 76]. In animal models, T increases cortical serotonin 2A receptor binding densities [77] and, in humans, cortical serotonin 2A receptors decrease with depression and aging [78].

3.2. T Treatment of Depression in Older Men. Although the practice of hormone replacement therapy began as long ago as the 18th century, with the use of extractions of reproductive organs of animals to treat a variety of ailments or to enhance the capacity for enjoyment of work and sexual activity [79–81], the role of T therapy for middle-aged and older men with depression is still uncertain (see Tables 3 and 4). T replacement in hypogonadal males generally decreases anger, nervousness, irritability and anxiety [35], and consistently leads to increased sexual interest and activity ([82, 83], see [84] for negative results).

In a randomized, placebo-controlled, double-blind, phase III trial (ClinicalTrials.gov identifier: NCT00696748), 184 men suffering from both the metabolic syndrome and hypogonadism were treated for 30 weeks with either parenteral testosterone undecanoate (TU; 1,000 mg IM TU) or placebo injections [85]. Depression was assessed at the baseline and at 18 and 30 weeks with the BDI. At baseline, depression significantly correlated with the total testosterone level ($r = -0.16$; $P = 0.03$). When comparing the changes over time in patients treated with TU versus the placebo group, there was a significant improvement in depression (mean difference versus placebo after 30 weeks: -2.5 points; 95% CI: -0.9 ; -4.1 ; $P = 0.003$). Effects were strongest in men with the lowest baseline total testosterone (< 222 ng/dL).

In a sample of 51 hypogonadal men (aged 22 to 60 years) studied for 60 days, T replacement improved positive mood parameters, such as energy, well-being and friendliness, and decreased negative mood parameters including anger, nervousness, and irritability [35]. Direct correlations between serum T and dihydrotestosterone (DHT) with mood scores were only observed in the baseline period, when serum androgen levels were below the normal range. This observation may indicate that it is possible that, once a minimally adequate serum T/DHT level is achieved by T replacement therapy, further increases in serum T/DHT levels do not further contribute to the improvement in mood variables. In a subsequent trial, Wang et al. [82] administered a transdermal T gel formulation to hypogonadal men (227 men aged 19 to 68 years) over a period of 180 days.

TABLE 3: Intervention studies in samples of patients with depressive symptoms.

Author, year	Type of study	Sample size	Age	Treatment	Outcome measures	Results
Wang et al. [35]	Pre-/postanalyses	51 hypogonadal men	22–60 yrs old	18 received T enanthate 200 mg im every 20 days, 16 received sublingual T cyclodextrin (SLT) at a dose of 2.5 mg three times daily, and 17 received SLT at a dose of 5.0 mg three times daily. The total treatment period was 60 days	Change in scores in single items on a 7-point Likert rating scale measuring anger, alertness, irritability, energy, sadness, tiredness, friendliness, nervousness, and well-being	T replacement led to significant decreases in anger, irritability, sadness, tiredness, and nervousness, and significant improvement in energy, friendliness, and well-being in all subjects as a group. Baseline serum T was positively correlated with friendliness and well-being, and negatively correlated with nervousness, irritability and tiredness. After T replacement these correlations disappeared.
Wang et al. [35]	Pre-/postanalyses	30 hypogonadal men	All 18+ years old	Sublingual T cyclodextrin 5 mg 3 times daily for 6 months	—	The patients were less nervous and more alert, friendly, and energetic during the 6-month treatment period compared with baseline.
Alexander et al. [107]	Cross-sectional	33 hypogonadal men receiving T replacement. 10 eugonadal men receiving T and 19 eugonadal men not administered T	Range: 19–60 yrs Mean: 41.1 yrs (hypogonadal); 33.4 yrs (eugonadal men receiving T); 32.7 (eugonadal men not administered T)	Eugonadal men received weekly i.m. injections of testosterone enanthate (TE) (200 mg). Hypogonadal men were treated either with 200 mg TE every 20 days or with 2.5 or 5.0 mg sublingual testosterone cyclodextrin 3 times daily	Change in POMS scores after 6 weeks of treatment.	T had positive effects on mood in hypogonadal men, but did not have any effects on mood in eugonadal men.
Wang et al. [82]	Double-blind RCT	227 hypogonadal men	About 3.9–11.0% of the subjects were <35 yrs, 23.3–36.8% were between 35–49 yrs, 55.1–57.5% were between 50–64 yrs, and 3.9–8.2% were 65+ yrs in the 3 initial treatment groups	In the first 3 months the subjects were randomized to receive 50 mg/day T gel in 5 g gel, 100 mg/day T gel in 10 g gel, or 2 nonscrotal patches delivering 5 mg/day (T patch). In the following 3 months, the subjects were administered 1 of the following treatments: 50 mg/day T gel, 100 g/day T gel, 5.0 mg/day T patch, or 75 mg/day T gel in 7.5 g gel	Sexual function and mood were assessed before clinic visits on day 0 and on days 30, 60, 90, 120, 150, and 180 during gel and patch application	All subjects as a group showed improvement in positive mood. Similarly, the negative mood summary scores showed significant decreases without showing between-group differences.
Pope et al. [93]	RCT	56 men	Range: 20–50 yrs	Testosterone cypionate for 6 weeks in doses rising to 600 mg/wk and placebo for 6 weeks, separated by 6 weeks of no treatment	Differences in YMRS and HAM-D scores	84% of those who received 600 mg/wk of testosterone cypionate exhibited minimal psychiatric effects (YMRS \leq 10), 12% became mildly hypomanic (YMRS= 10–19), and 4% became markedly hypomanic (YMRS \geq 20). The HAM-D remained low, with no changes during T administration or withdrawal.

TABLE 3: Continued.

Author, year	Type of study	Sample size	Age	Treatment	Outcome measures	Results
O'Connor et al. [108]	RCT	30 healthy men and 8 hypogonadal male patients	Healthy males: Range: 19–45 yrs; Mean: 28.2 yrs Hypogonadal men: Range: 23–40 yrs; Mean: 30.8 yrs	15 eugonadal-men received 200 mg i.m. T enanthate once weekly for 8 weeks, 15 received 200 mg i.m. 0.9% sodium chloride solution weekly for 8 weeks. The hypogonadal group received 200 mg i.m. T enanthate biweekly for 8 weeks	Differences in depression-dejection dimension of the POMS	Significant main effects were found for time, group, and for time x group interaction. Multiple comparisons found that the significant group effect was accounted for by significantly higher levels of total mood disturbance in the hypogonadal group than the eugonadal-treated and eugonadal-placebo groups. However, there was a significant reduction in total mood scores in the hypogonadal group by weeks 1-2 explaining the significant interaction effect.
Almeida et al. [69]	Pre-/postanalyses	40 men with prostate cancer treated with androgen blockade therapy	Range: 44–83 yrs; Mean = 72.4 yrs	Androgen blockade therapy (flutamide and leuprolide) for 36 weeks and subsequently followed up for another 18 weeks after discontinuation	Change in BDI scores	BDI scores increased significantly during the active treatment and declined somewhat thereafter. However, the number of people with clinically significant depressive symptoms did not change significantly.
Schmidt et al. [95]	Double-blind RCT	31 healthy adult men with no history of psychiatric illness or substance or anabolic steroid abuse	Range: 18–45 yrs; Mean: 30.8 ± 5.8 yrs	Leuprolide acetate (Lupron) 7.5 mg im every 4 weeks for 3 months. After the first month of Lupron alone, all men received (in addition to Lupron) testosterone enanthate (200 mg i.m.) or placebo every 2 weeks for 1 month each in a crossover design.	Changes in BDI scores	BDI scores significantly increased during Lupron plus placebo compared with baseline and Lupron plus testosterone.
Kenny et al. [102]	RCT	11 men with early cognitive decline and bioavailable T levels below 128 ng/dL	Range: 73–87 yrs; Mean: 80 ± 5 yrs	intramuscular testosterone (200 mg every 3 weeks) or placebo for 12 weeks	Changes in GDS scores	No significant changes were found in depression following T supplementation
Haren et al. [90]	Double-blind RCT	76 healthy men with at least two symptoms on the ADAM, a FT index (FTI) of 0.3–0.5 and TT greater than 8 nmol/L	Range: 60–86 yrs Mean: 68.5 ± 6 yrs	80 mg twice daily of testosterone undecanoate—TU (39 subjects) or identical placebo (37 placebo) for 12 months	Differences in GDS scores	From baseline to month-6 there was a significant effects of treatment on depression. No clinically relevant differences on the GDS between the testosterone and placebo group.

TABLE 3: Continued.

Author, year	Type of study	Sample size	Age	Treatment	Outcome measures	Results
Gray et al. [94]	RCT	60 healthy men	Range: 60–75 yrs	Monthly injections of long-acting GnRH agonist to suppress endogenous T production and randomization to one of five doses (25, 50, 125, 300, and 600 mg) of testosterone enanthate weekly for 20 weeks	Changes in HAM-D and YMRS	Baseline depression and mania were not correlated with log FT levels. Changes in mood did not differ by group and were not significantly correlated with FT or TT.
Giltay et al. [85]	Double-blind RCT	184 men with TT below 12.0 nmol/L or FT below 225 pmol/L, and a diagnosis of the MetS	Range: 35–69 yrs; Mean: 52.1 ± 9.6 yrs	30 weeks with either parenteral testosterone undecanoate (1,000 mg i.m., at baseline, and after 6 and 18 weeks) or placebo injections	Association between BDI and TT. Changes in BDI scores	At baseline, BDI scores significantly correlated with TT ($r = -0.16$). More improvements in BDI for those treated with T (mean difference versus placebo after 30 weeks: -2.5 points).
Aloisi et al. [88]	Open label	9 opioid-induced hypogonadic men, T less than 2-3 ng/mL in at least two determinations in the previous 3-4 months	Range: 38–74 yrs; Mean: 59.0 ± 4.4 yrs	One-month supply of testosterone gel, a hydroalcoholic compound containing 50 mg testosterone in 5 g gel in each sachet for 1 year	Change in CES-D scores from baseline	CES-D showed no significant change from baseline to follow-up assessments at 3, 6 and 12 months.

T: testosterone; BT: bioavailable testosterone; FT: free testosterone; DHEA: dehydroepiandrosterone; SHBG: Sex hormone-binding globulin; BMI: body mass index; HAM-D: Hamilton scale for depression; BDI: Beck depression inventory; CES-D: center for epidemiologic studies depression scale; GDS: geriatric depression scale; YMRS: young mania rating scale; MDD: major depressive disorder; MetS: metabolic syndrome.

TABLE 4: Intervention studies in samples of patients with depressive disorders.

Author, year	Type of study	Sample size	Age	Treatment	Outcome measures	Results
Seidman and Rabkin [98]	Open-label	5 depressed men who had low T levels and had not responded to an adequate SSRI trial	Range: 34–50 yrs; Mean: 40 ± 5.9 yrs	400 mg testosterone replacement biweekly for 8 weeks	Changes in HAM-D scores from baseline	Significant recovery from major depression following T augmentation.
Perry et al. [97]	RCT	15 elderly eugonadal males with MDD (HAM-D > 18)	Mean: 61.3 ± 7.6 yrs	Following a single-blind 2-week placebo lead-in, patients were randomly assigned to treatment with either a physiologic dose of testosterone cypionate (TC), 100 mg/week, or supraphysiologic dose of 200 mg/week i.m. for 6 weeks	Changes in HAM-D scores	42% decrease in the mean HAM-D scores. However, the majority of the change was due to improvement in the late-onset depression patients. The TC dose did not affect the response.
Pope et al. [101]	Double-blind RCT	22 MDD patients with morning serum TT of 350 ng/dL or less who were receiving antidepressant treatment	Range: 30–65 yrs; Mean: 48.9 ± 8.5 (T group) and 49.5 ± 9.8 (placebo group)	12 men received 1% testosterone gel (10 g/day) and 10 received placebo	BDI, HAM-D, CGI-S	Subjects receiving testosterone gel had significantly greater improvement on the HAM-D than subjects receiving placebo. These changes were noted on both the vegetative and affective subscales of the HAM-D. A significant difference was also found on the CGI-S but not on the BDI.
Orengo et al. [99]	RCT	12 hypogonadal men who were receiving antidepressants (on appropriate dose) for a minimum of 6 weeks	Range: 52–80 yrs; Mean: 63 ± 8.5 yrs	Placebo or active T gel 1% at a dose of 5 g for 24 weeks	Differences in HAM-D scores	There was a significant improvement in HAM-D at 12 weeks of testosterone treatment as compared to baseline. However, there was no statistical difference between placebo and testosterone treatments.
Seidman and Roose [84]	Double-blind RCT	30 men with low and low-normal T levels (i.e., total T <or = 350 ng/dL) and MDD	Range: 33–71 yrs; Mean: 52 ± 8 yrs	Weekly intramuscular injections of either T enanthate 200 mg or placebo for 6 weeks	Differences in HAM-D scores	The HAM-D scores decreased significantly in both T and placebo groups, and there were no significant between-group differences.
Shores et al. [86]	Double-blind RCT followed by an open-label extension phase	33 men with TT levels of ≤280 ng/dL and subthreshold depression (dysthymia or minor depression, according to DSM-IV)	All 50+ yrs old; Mean: 57.1 ± 5.7 yrs (T); 61.7 ± 7.0 yrs (placebo)	Either 7.5 g of testosterone gel (16 men) daily for 12 weeks, followed by a 12-week open-label extension phase during which all subjects received 7.5 g of testosterone gel	Differences in HAM-D scores	At the end of the double-blind phase, testosterone-treated men had a greater reduction in HAM-D scores and a higher remission rate of subthreshold depression (52.9% versus 18.8%) than did placebo-treated men. At the end of the extension phase, there were no significant between-group differences in the remission rate of depression between the original testosterone group and the original placebo group (58.8% versus 68.8%, resp.).

TABLE 4: Continued.

Author, year	Type of study	Sample size	Age	Treatment	Outcome measures	Results
Seidman et al. [87]	Double-blind RCT	23 men with dysthymic disorder and with low or low-normal T level (i.e., TT < 350 ng/dL)	Mean: 50.6 ± 7.0 yrs	200 mg of testosterone cypionate im or placebo every 10 days for 6 weeks	Difference in HAM-D and CGI-I	HAM-D score decreased significantly more in the T group (7.46 ± 4.56) than in the placebo group (1.8 ± 4.13). Patients in the T group were more likely to remit (53.8% versus 10%) than patients in the placebo group.
Pope et al. [91]	Double-blind RCT	100 medically healthy adult men with MDD showing partial response or no response to an adequate SSRI trial during the current episode and a screening TT ≤ 350 ng/dL	Mean: 50.6 ± 8.2 and 49.9 ± 7.1, respectively for those treated with T and those in the placebo group	Placebo gel (50 men) or testosterone gel (50 men) at 5 g/day. If the testosterone level at week 1 exceeded the physiologic range (91070 ng/dL), the investigator reduced the dose of gel to 2.5 g/day; if the level was 500 ng/dL or lower, then the investigator issued instructions to raise the dose to 10 g/day	Difference in HAM-D and MADRS	No significant difference in the antidepressant effects of T and placebo gel augmentation

T: testosterone; BT: bioavailable testosterone; FT: free testosterone; DHEA: dehydroepiandrosterone; DHEAS: dehydroepiandrosterone sulphate; SHBG: Sex hormone-binding globulin; BMI: body mass index; HAM-D: Hamilton scale for depression; BDI: Beck depression inventory; CES-D: center for epidemiologic studies depression scale; GDS: geriatric depression scale; POMS: profile of mood states; YMRS: young mania rating scale; CGI-S: clinical global impression scale-severity; CGI-I: clinical global impression scale-improvement; MDD: major depressive disorder; MDD: major depressive disorder; Mets: metabolic syndrome.

Mood improved and the improvement was maintained with continued treatment.

Recently, Shores et al. [86] examined the effect of testosterone treatment in older, hypogonadal men (50+ years old) with subthreshold depression in a double-blind randomized controlled trial. Participants received either 7.5 g of testosterone gel or placebo gel daily for 12 weeks, followed by a 12-week open-label extension phase during which all subjects received 7.5 g of testosterone gel. At the end of the double-blind phase, testosterone-treated men had a greater reduction in depression ($P < 0.05$) and a higher remission rate of subthreshold depression (52.9% versus 18.8%, $P < 0.05$) than did placebo-treated men. At the end of the open-label phase, the testosterone group had sustained improvement, while patients who had received placebo in the previous 12 weeks improved, and there were no differences between groups on the number of depressive symptoms reported.

Seidman et al. [87] conducted a six-week double-blind placebo-controlled clinical trial in 23 men with mid-life onset male dysthymic disorder and with low or low-normal testosterone level (TT < 350 ng/dL). After the intervention, the depression decreased significantly more in the testosterone group than in the placebo group ($P < 0.01$).

However, some studies have shown that, in the short and long term [88], T replacement is not superior to placebo in elderly men with low-normal gonadal status, or in men with the lowest BT levels [89–91].

Androgen treatment in eugonadal men has demonstrated subtle changes in sexual arousal, cognition, and mood [36], with a significant increase in manic and aggressive symptoms [92, 93]. However, two studies failed to observe effects of T on mood in healthy men with induced hypogonadism who were given T [94, 95].

To date, little evidence supports the use of androgen therapy in older depressed men [96]. In a study by Perry et al. [97], a subgroup of elderly depressed males (aged 70 and over) improved with T therapy. In a study of 15 elderly eugonadal males with major depressive disorder (MDD, according to the DSM-IV criteria), 5 with early onset MDD, and 10 with late onset MDD, treatment with T cypionate (100 mg/week or 200 mg/week IM for 6 weeks) was efficacious only in some cases of late-onset depression.

Androgen administration in open and blind clinical trials to chronically depressed men or to hypogonadal men with depression refractory to selective serotonin reuptake inhibitors (SSRIs) improved depressive symptoms. Human and animal studies have demonstrated that T treatment may facilitate the antidepressant drug response [98–100]. T augmentation in men with major depression refractory to SSRIs treatment and low or borderline TT levels (200–350 ng/dL) produced significant positive results in short-term treatment (12 weeks) [98, 101], but doubts arose about longer-term treatment (20 weeks) [99].

At the present time, available data do not suggest the use of T in the treatment of depression in PADAM. Data on older men suffering from depression and PADAM are still few and inadequate [102], and the current clinical guidelines for men

with low serum T concentration stress that T therapy for depression is irrelevant [12, 103, 104].

Furthermore, T supplementation may be associated with some adverse effects, such as erythrocytosis, acne and oily skin, detection of subclinical prostate cancer, growth of metastatic prostate cancer, and reduced sperm production and fertility [12, 104]. Other, uncommon, adverse events for which there is weak evidence of association with testosterone administration are gynecomastia, male pattern balding (familial), and induction or worsening of obstructive sleep apnea. Formulation-specific adverse effects include fluctuation in mood or libido, pain at injection site, excessive erythrocytosis (especially in older patients), and coughing episodes immediately after the intramuscular injection for intramuscular injections of testosterone enanthate, cypionate, or undecanoate, as well as frequent skin reactions at application site for transdermal patches, and potential risk for testosterone transfer to partner or others in close contact with the individual, and skin irritation for transdermal gel [12].

4. Discussion

As Western populations represent an aging society with continuing gains in life expectancy [4], hypogonadism in older men may have significant public health implications [67, 109, 110]. For example, over the last decade, this has led to a significant market growth in T therapies for men 40 years and older [4].

PADAM includes behavioral and depressive symptoms that vary greatly from individual to individual, being the result not only of biological and psychosocial changes, but also of personal ability to adapt to such changes. The efficacy of T therapy in the treatment of depression in elderly hypogonadal men is inconclusive. Research on T replacement therapy for depressive symptoms of PADAM reveals the great variability of the results.

Nonetheless, androgens supplementation may be a useful as adjunctive therapy in depressed hypogonadal men. Several study reported that antidepressants may be associated with sexual dysfunction in adult patients [111], and up to 20% of users may suffer from sexual dysfunction [112]. Sexual dysfunction may also be associated with discontinuation of antidepressants treatment [113]. T treatment may have beneficial effects on sexual functions [83, 89, 94, 103, 114–116]. Recently, Amiaz et al. [83] conducted a 6-week, double-blind, placebo-controlled clinical trial of testosterone gel versus placebo gel in men with MDD who were currently taking a serotonergic antidepressant and exhibited low or low-normal testosterone level. The results indicated that those taking testosterone improved in sexual functions as measured through the International Index of Erectile Function more than those in the placebo arm. Furthermore, the results indicated that the improvement in sexual functioning did not appear to be attributable to improvement in depression.

T treatment may be particularly useful to improve quality of life in elderly hypogonadal men, because its effect on muscular strength [117–119] and may be on cognitive functions [57, 120–122].

However, due to adverse effects associated with T therapy, pretreatment screening for parameters related to potential risks of testosterone supplementation is essential. T supplementation is contraindicated in individuals with hematocrit of 52% and over [123, 124], prostatic carcinoma, an androgen sensitive-tumor, and in cases of mammary carcinoma in men [12, 103].

In conclusion, despite the causative nature of the relationship between low T levels and depression is uncertain, many hypogonadal men suffer from depression and vice versa. Supplementation with testosterone failed to show sound evidence of effectiveness in the treatment of depression. Nevertheless, T supplementation has proved to be effective on some domains significant for the quality of life of patients with PADAM. Those effects may partially mediate the effects on depressive symptomatology reported in some trials. Thus, the overall improvement in well-being and health, related to the quality of life in aging males with partial androgen deficiency, may have a positive impact on their mood.

Conflict of Interests

The authors declare that there is no conflict of interest associated with this manuscript.

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Review Article

Muscular Dystrophies at Different Ages: Metabolic and Endocrine Alterations

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Common metabolic and endocrine alterations exist across a wide range of muscular dystrophies. Skeletal muscle plays an important role in glucose metabolism and is a major participant in different signaling pathways. Therefore, its damage may lead to different metabolic disruptions. Two of the most important metabolic alterations in muscular dystrophies may be insulin resistance and obesity. However, only insulin resistance has been demonstrated in myotonic dystrophy. In addition, endocrine disturbances such as hypogonadism, low levels of testosterone, and growth hormone have been reported. This eventually will result in consequences such as growth failure and delayed puberty in the case of childhood dystrophies. Other consequences may be reduced male fertility, reduced spermatogenesis, and oligospermia, both in childhood as well as in adult muscular dystrophies. These facts all suggest that there is a need for better comprehension of metabolic and endocrine implications for muscular dystrophies with the purpose of developing improved clinical treatments and/or improvements in the quality of life of patients with dystrophy. Therefore, the aim of this paper is to describe the current knowledge about of metabolic and endocrine alterations in diverse types of dystrophinopathies, which will be divided into two groups: childhood and adult dystrophies which have different age of onset.

1. Introduction

There are about 30 different types of muscular dystrophies caused by alterations in diverse genes, which are characterized by the progressive loss of muscle in accordance with age of onset, severity, and the group of muscles affected [1]. The altered protein in most of these dystrophies is located in muscle fiber and is linked to other proteins, enzymes, or extracellular matrix [2]. Myopathologies are associated with different ages of onset, for example, Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) as well as Emery-Dreifuss muscular dystrophy (EDMD) demonstrating their first clinical manifestations during childhood [3], whereas some laminopathies such as myotonic dystrophy or limb-girdle muscular dystrophy are developed during adulthood [4]. This group of diseases can cause different physical symptoms such as contractures and scoliosis, respiratory impairment, swallowing and feeding difficulties, and, in some cases, metabolic alterations have been reported

[4, 5]. Even so, the primordial clinical sign is muscle weakness [4, 5].

Skeletal muscle is responsible for 70–80% of whole body insulin-stimulated glucose uptake, disposal, and storage; therefore, this tissue is considered the major “player” in energy balance [6]. Furthermore, skeletal muscle has influence on the metabolism and storage of lipids and plays an important role in hormone signaling in insulin sensitivity [6]. Insulin receptors are localized in the cell membrane of target tissues (liver, adipose tissue, and muscle fibers) [7, 8]. It is important to emphasize that in dystrophinopathies the cell membrane of myocytes is damaged [3]. We have hypothesized that dysfunction of membrane of myocytes could alter the insulin receptor function, and as a result an increase may occur in the risk for developing insulin resistance. This fact will be discussed later. Insulin resistance is an important factor for the development of type II diabetes and a risk factor for cardiovascular disease, dyslipidemia, hypertension, and obesity [6, 9]. The progression of muscular damage depends

on protein mutation [3]. However, it is important to consider that muscle damage may be age related. The aim of this paper is to describe the current knowledge about of metabolic and endocrine alterations in diverse types of dystrophinopathies, which will be divided into two groups: childhood and adult dystrophies which have different age of onset.

2. Childhood Dystrophies

2.1. Clinical Characteristics of Duchenne/Becker Muscular Dystrophy. DMD has an incidence of 1/3500 live male births [10]. Initial symptoms begin in early infancy, presenting with muscular weakness between 2 and 4 years of age [11]. This appears when the patient begins to walk [12]. These hip weaknesses will be manifested in an awkward walk with a tendency to fall; later, stair climbing becomes difficult [12]. A classic sign of muscular weakness is Gower's sign, which indicates weakness of the pelvic girdle muscles [11, 12]. Usually, joint contractures at the ankles and hips are increased as the disease progresses [11, 12]. Finally, the development of muscle atrophy leads to wheelchair dependency, which typically occurs between 9 and 12 years of age [10]. The susceptibility for lung infections increases and respiratory capacity decreases [13]. Respiratory insufficiency appears at ~14 years of age and may result in death at ~25–30 years of age [13]. Another cause of death may be cardiomyopathy, which begins at ~5 years of age and evolves in a parallel manner with progression of the disease until this condition eventually leads to the patient's death [14]. Even so, cardiomyopathy is responsible for ~60–80% of deaths [14].

Eagle et al. [15] reported a mean age of death of 14.4 years in the US during the decade of the 1960s, and this value increased to 25.3 years of age in 1990. Fortunately, advances in the treatment of dystrophinopathies have improved life expectancy because patients may reach adulthood in the third and in some cases the fourth decade of life [15].

Becker muscular dystrophy (BMD) is clinically similar to DMD but is a less severe form of myopathy, affecting 1/30,000 males [16]. Patients with BMD start to show clinical signs between 2 and 20 years of age. Compared to DMD, progression in BMD is slower [17, 18]. Some BMD patients present clinical signs similar to those of DMD, whereas some patients are still able to walk at the age of 60 years [19]. Inability to walk prevails at about 30 years old, and death is frequently present 30 years after the appearance of the first clinical signs [20]. Cardiomyopathy usually occurs in 73% of BMD patients >40 years old [20].

Symptoms of Emery-Dreifuss muscular dystrophy (EDMD) generally appear during the first decade of life [21]. The principal clinical characteristics are contractures of the neck extensor muscle, spine, Achilles tendons, and elbows [22]. After muscle weakness, wasting appears during the end of the second decade of life and begins in a humeroperoneal distribution [23]. Cardiac complications begin at the end of the second decade, and sudden cardiac death due to ventricular dysrhythmia is common in this dystrophy [24].

DMD and BMD are X-linked diseases caused by mutations in the DMD gene, which is responsible for encoding dystrophin protein and is located at locus Xp21 [25–27].

Dystrophin protein is associated with an oligomeric protein complex known as dystrophin-glycoprotein complex or dystrophin-associated protein complex [28, 29]. The mechanical function of the dystrophin-glycoprotein complex is to stabilize the plasma membrane (sarcolemma) during the stress of repeated contraction and relaxation cycles [30]. In patients with DMD, dystrophin is absent in the sarcolemma, whereas in BMD its expression is greatly reduced but is still located in the sarcolemma [31]. Mutations (deletions, duplications, point mutations) cause the lack or semilack of dystrophin [32]. These mutations have many functional and structural consequences in skeletal muscle, in DMD patients; muscle biopsy characteristically demonstrates necrotic or degenerating muscle fibers [32]. These necrotic fibers are surrounded by macrophages. Small immature centrally nucleated fibers are also observed, reflecting muscle regeneration from myoblasts [31–33] that results in a balance between necrotic and regenerative processes in the early phase of the disease. Later, the regenerative capacity of the muscles appears to be exhausted, and muscle fibers are gradually replaced by connective and adipose tissue (Figure 1) [33].

EDMD is usually inherited as an X-linked recessive disorder, although an autosomal dominant form has also been described [34]. This dystrophy is caused by mutations in the gene for emerin (EMD gene) localized on chromosome Xq28 [35]. Emerin is an integral serine-rich protein of the nuclear envelope inner membrane, which is ubiquitously expressed in most tissues and contains 254 amino acids [36]. The autosomal form of EDMD is caused by mutations in the LMNA gene localized at chromosome 1q21.2-q21.3, which encodes an A-type nuclear lamin [37]. This lamin is an intermediate filament protein associated with the inner nuclear membrane [38]. Emerin interacts with both lamin A and lamin C in the nucleus. It has been proposed that these mutations may result in increased nuclear fragility during mechanical stress or increased susceptibility to apoptosis [39].

2.2. Endocrine System. The most important endocrine alteration in DMD/BMD patients is hypogonadism, which has been related to dystrophies [40]. Consequences of this state are delayed puberty, growth failure, osteoporosis, and metabolic abnormalities [40–42]. In 2008, Al-Harbi et al. [40] measured total and free serum testosterone levels in 59 men with different dystrophinopathies. Results obtained showed that 54% had low total testosterone, 39% had low total and free values, and 8% had low free with normal total levels [40]. In addition to Becker and Duchenne dystrophies ($n = 12$), other dystrophinopathies were included such as monotonc dystrophy ($n = 12$), facioscapulohumeral dystrophy ($n = 11$), metabolic myopathy ($n = 7$), and body myositis ($n = 17$) [40]. Interestingly, there were no significant differences in the prevalence of hypogonadism among the various forms of myopathy, even after considering age as a confounder [40]. It has been established that testosterone levels decline with aging, and this is associated with decreased muscle mass and strength in healthy subjects [43]. Testosterone treatment increases muscle mass and strength in older

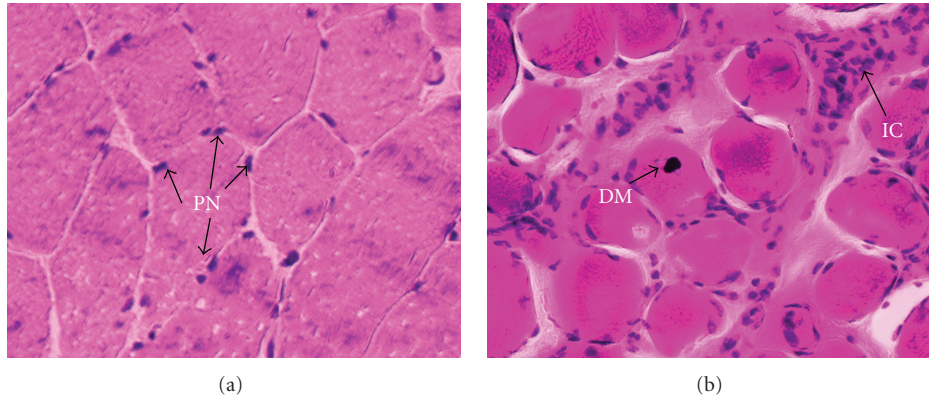


FIGURE 1: Histology of necrotic skeletal muscle. Image (a) showed a normal skeletal muscle from quadriceps muscle, characterized by healthy myofibers with peripheral nuclei (PN). Skeletal muscle of control subjects was obtained from males without dystrophinopathies at 40 years old. Image (b) showed a necrotic skeletal muscle from quadriceps muscle of a patient with DMD/DMB at five years old, characterized by infiltrating inflammatory cells (ICs) and degenerating myofibers (DMs). Transverse muscle sections stained with haematoxylin and eosin. Scale bar represents 50 μ m. This biopsy was obtained for the purpose of performing diagnostic.

hypogonadal males [44]. The importance of testosterone in the maintenance of muscle mass is critical, and testosterone therapy should be considered when hypogonadism is present [43, 44].

Growth hormone (hGH) has anabolic effects in normal skeletal muscle [45]. It has been suggested in only one study that this hormone plays a role in the pathogenesis of DMD, but there is insufficient evidence to support that idea [45, 46]. For instance, in one study, treatment with hGH was administered to DMD patients, but no effect was shown on clinical status and natural history of DMD, either beneficial or detrimental [47]. In addition, Merlini et al. [45] demonstrated in DMD patients with impaired hGH secretion that no association exists between diminished secretion of hGH and different forms of the disease. Interestingly, there is a case report of hGH treatment of a young male with DMD with hGH deficiency who showed improved growth velocity and motor function [45]. Controversies still exist about the benefit and the role of hGH and the resolution in regard to treatment administration with hGH for counteracting short stature. This treatment should be individualized with the objective to obtain improved results [40].

No effective treatment has yet been demonstrated to ameliorate the various consequences of muscular dystrophies [48]. However, in recent years a range of approaches have been developed to correct the genetic defect, restore functional expression of dystrophin, slow disease progression, and improve the quality of life for DMD patients [49]. Those treatments can be categorized into three classes: genetic, cell-based, and pharmacological approaches such as corticosteroids [50].

The use of pharmacological treatment with corticosteroids is justified by the fact that DMD is characterized by aggressive inflammation, and there is strong evidence that this contributes to myofiber necrosis [48, 51]. Until a cure for DMD is found, treatment will involve administration of corticosteroids combined with interventions to alleviate cardiac and respiratory problems [51].

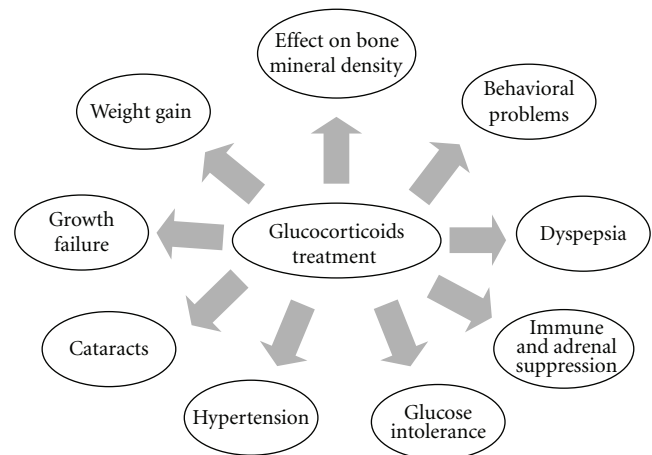


FIGURE 2: Side effects of glucocorticoid treatment in muscular dystrophies.

However, it is important to note that corticosteroids have a catabolic effect on muscle (nonexercised muscle) and have an effect on preserving existing muscle fibers and reducing inflammation, even though their exact mechanism of action in dystrophic skeletal muscle is unknown [12, 48]. Moreover, use of glucocorticoids [GC] in patients with DMD and BMD is common, but GCs have different side effects (Figure 2) [48]. Two of the most important side effects are growth failure and delayed puberty [52]. Currently, there are no studies examining the outcomes of treating delayed puberty in patients with DMD.

2.3. Metabolic Alterations. About a decade ago, clinical researchers began to notice that patients with DMD tended to present obesity [53]. However, it was necessary to demonstrate the prevalence of obesity in children with DMD because obesity had only been described from clinical experience [54]. For this reason, it was also necessary to measure

body composition of these patients which, in turn, depended on technology available [55].

With the advance in knowledge, it was observed that DMD patients also develop malnutrition as a consequence of the progression of the illness [56]. In the following paragraphs, some metabolic issues will be described such as malnutrition and obesity and, additionally, some abnormalities in glucose metabolic pathway.

2.3.1. Malnutrition. In patients with dystrophinopathies, nutrition is a problem to consider. It has been reported that 50% of DMD patients are underweight by the age of 18 [54]. Some studies have shown that this problem generally is caused by feeding difficulties, gastrointestinal dysfunction, and reduced weight gain [57]. It has also been observed that chewing and swallowing difficulties in both ambulant and nonambulant DMD patients are related to increased weakness of masticatory muscles, malocclusion, and other abnormalities of the oropharyngeal process [58]. Additionally, gastric distension has been reported in DMD and BMD, which can contribute to delay gastric emptying, gastroesophageal reflux, and subsequent nutritional disturbances [59].

Malnutrition has also been observed in patients with DMD/BMD who are severely compromised by respiratory failure [13]. This fact has been described in advanced stages of those dystrophinopathies when breathing effort increases and, as a consequence, caloric requirements drastically increase. Gonzalez-Bermejo et al. [13] showed that specific nutritional measures should be taken when patients have advanced forms of dystrophy, and mechanical ventilation becomes necessary because, surprisingly, these patients have balanced energy intakes and resting energy expenditure (REE); hence, they are not likely to suffer from significant malnutrition. However, Gonzalez-Bermejo et al. [13] found that 34% of patients aged 25 ± 4 years who had received nocturnal mechanical ventilation had a decrease of REE. This effect was also observed in patients with DMD of different age groups (10–11 years; 12–14 years; 15–17 years; 18–29 years), where REE was significantly lower than the value obtained for healthy controls [13]. Both the low REE and the low physical activity during the early teenage years result in a low energy requirement and may be related to obesity that frequently occurs in this age group [13]. In contrast, in later stages of the disease, patients increase their basal physical activity [60]. It is possibly due to the presence of respiratory failure, may lead to a high energy requirement, and thus becomes one of the risk factors for development of malnutrition [60]. Additionally, it is important to consider that loss of the ability to self-feed in DMD/BMD patients is very common [61]. The length of meal time also increases with age, ranging from a mean of 18 min in younger patients to a mean of 32 min in older patients [62]. The increase in time is probably related to a combination of increasing weakness of the masticatory muscles and difficulties in chewing and swallowing [62]. Perhaps it would be a good choice to introduce dietary modifications as cutting food into smaller pieces and changing the texture to soft foods in order to facilitate chewing and to reduce meal time [62].

2.3.2. Obesity. As previously mentioned, obesity is an important identified problem in patients with dystrophinopathies [53]. Body mass index (BMI) is the most frequently used indicator in clinical practice in order to make the diagnosis of overweight or obesity [63]. Nonetheless, the main limitation of the BMI is that it does not discriminate between fat mass and lean mass [64]. It has been observed that individuals with a BMI within normal limits have fat mass measurements that fall within values considered as obesity when these have been measured with more precise methods [64]. Therefore, it is necessary to measure fat mass through body composition. It is possible to evaluate the general nutritional status by body weight composition, which could be estimated by measures such as skinfold thickness (ST), dual energy X-ray absorptiometry (DXA), bioelectrical impedance analysis (BIA), and magnetic resonance imaging (MRI) [65].

At the age of 7 years, obesity may occur in patients with DMD, and by the age of 13 years prevalence is 54%, and distribution of body fat is centralized [54]. Moreover, Martigne et al. [56] provided additional information about nutritional status in DMD patients during different ages. This group of investigators studied the progression of nutritional status in 17 DMD patients born prior to 1992. Obesity was evaluated using Griffiths & Edwards charts. According to it, obesity was defined by body weight/age ratio $\geq 151\%$. At the age of 13 years, those patients showed a prevalence of 73% for obesity and 4% for underweight. At age 15–26 years, the prevalence of obesity decreased to 47% and prevalence of underweight increased to 34%. Also, obesity at the age of 13 years was associated with later obesity, whereas normal weight status and underweight in 13-year-old patients were predicted later [56].

It has been observed that obesity contributes to the progression of the disease by exerting extra force on already weakened muscle groups, essentially decreasing mobility [56]. Obesity also has implications on increased respiratory involvement as well as poorer psychosocial development [57]. There is one hypothesis that describes the positive association of loss of ambulation and obesity increment in DMD patients [12, 13]. Although Martigne et al. [56] found no relation between the age of ambulation loss and development of obesity.

Generally, fat mass is higher in patients with dystrophy than in healthy subjects [66]. Indeed, as the dystrophic process advances with age, percentage of fat mass increases at least during the first two decades of life [67]. In fact, body composition of DMD patients has been evaluated (by bioelectrical impedance analysis and skinfold-thickness measurement), and the results show that lean mass in DMD (65.3%) was lower (between $\sim 12\%$ and 25%) than in healthy children, whereas fat mass in DMD (31.9% versus 22%) was higher than in control children [67]. Additionally, a positive correlation was found between age and percentage of fat mass and a negative correlation between age and percentage of lean mass [67]. The authors suggest that muscle may have been replaced by connective tissue [67]. In this context, Zanardi et al. [55] studied obese and nonobese children with DMD and evaluated the alteration of body composition in boys with DMD using magnetic resonance imaging. Only obese

children showed a markedly increased fat mass (>50%), whereas nonobese children with DMD showed fat mass values similar to healthy boys [55]. In both obese and nonobese children with DMD, the characteristic that was dramatically reduced was muscle mass, showing a reduction of 27% of muscle volume compared with control subjects [55].

It is important to emphasize that the advance in the knowledge and development of new technologies for assessing nutritional status has shown that patients with DMD present abnormal nutritional status such as obesity or malnutrition as part of the natural course of DMD. These alterations are related to advancing age because dystrophy progresses over the course of time.

2.3.3. Glucose and Insulin Metabolism. To date, relatively little is known about metabolic alterations in patients with DMD and BMD. However, there are studies that described some disturbances in glucose metabolism, which include reduced glycolytic substrates, reduced activity of glycolytic enzymes, and defects in insulin receptor signal transduction (Figure 3) [68].

Some studies have suggested that there are metabolic differences in the skeletal muscle of patients with DMD and healthy subjects [69]. For example, it has been proven that the concentration of glycolytic substrate glucose, gluconeogenic amino acids such as glutamine and alanine and lactate, a glycolytic product, is lower in DMD patients as compared to controls in skeletal muscle [70]. Also, the decrease in the concentration of lactate in the muscle of DMD patients may be due to the reduction in anaerobic glycolytic activity or lower substrate concentration [71]. As a result, the low concentration of glucose metabolism substrates may be one of the reasons for energy deficit in DMD patients [72].

Nishio et al. also showed that glucose serum concentration in DMD patients is significantly lower and is associated with low creatine kinase activity. This fact may probably be one of the causes of energy deficit in DMD patients [72]. These findings also have a relationship with other causes described in different studies in which glycolytic enzymes were found to have reduced activity in lactate dehydrogenase, aldolase, and pyruvate kinase from muscle biopsies of DMD patients, supporting reduced anaerobic glycolytic activity [68]. Those results are in agreement with the low concentration of previously described glucose metabolism substrates.

Although there are no reports describing whether DMD patients present insulin receptor signal transduction alterations, some studies have evaluated whether there is a defect in insulin secretion or receptor [73]. These studies hypothesized that the possible changes in the sarcolemma of skeletal muscle cells could produce defects in insulin signaling [73]. This has been proposed because patients with diabetes present skeletal muscle weakness that could lead to changes in the sarcolemma as a result of defects in insulin receptor internalization and processing that have been well described in insulin resistance and diabetes [74]. Further research is needed to clarify this hypothesis.

There are other types of evidence that have demonstrated damage of the plasma membrane of myocytes of boys with

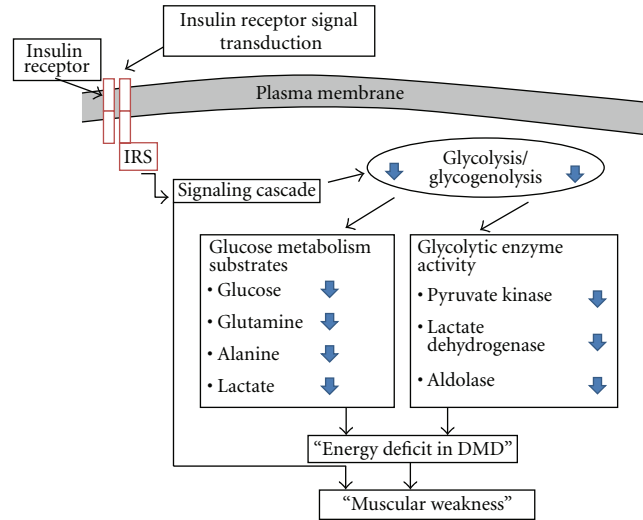


FIGURE 3: Some aspects of impaired glucose metabolism in patients with childhood muscular dystrophies. The damage in the glucose metabolism, no matter the reason, may probably be one of the causes for energy deficit in DMD patients, and this low energy could result in muscular weakness in muscular dystrophies.

DMD and BMD [75]. It is well known that membrane properties depend on phospholipid composition [76]. A significant reduction has been observed in muscle biopsies of DMD patients in some membrane components or phospholipids such as total creatine, glycerophosphorylcholine, phosphoryl choline, carnitine, choline, and acetate [68], and lower levels of choline-containing compounds indicate membrane abnormalities. Additionally, a lower ratio of trimethyl amides (TMAs) compared with healthy tissue has been demonstrated in biopsies of DMD patients. TMAs are constituents of phospholipid metabolism and cell membranes, and decreased TMA is considered to be associated with a lower number of cells and reduced rate of membrane synthesis [77]. Decrease in TMA may reflect degenerative changes in the muscles of patients with DMD, resulting in alterations in signal transduction that take place in the sarcolemma [77].

However, despite the above-mentioned evidence, scarce information exists about the alterations in glucose and insulin metabolism in DMD patients. There is only one study by Freidenberg and Olefsky in which an oral glucose tolerance test and the measurement of insulin binding on erythrocytes were performed in DMD patients and age-matched healthy males. The results of this study showed that insulin binding in erythrocytes was 20–30% lower in DMD patients than in healthy subjects. This difference indicated a lower affinity of insulin to its receptor in erythrocytes in DMD patients. This alteration may be present prior to the development of insulin resistance, which may occur in severely immobilized patients. One possible cause for this fact is the increased progression of the dystrophy. Furthermore, in the same study, it was found that patients with DMD had elevated levels of glucose and insulin in comparison to a healthy control group [73].

TABLE 1: Clinical aspects, metabolic and endocrine issues on muscular dystrophies.

		Age of onset	Clinical characteristics	Metabolic issues	Endocrine issues	Clinical complications	Life span
Childhood Muscular Dystrophy	DMD	Early infancy	Muscle weakness Gower's sign Joint contractures Muscle atrophy	Obesity Insulin resistance Malnutrition	Hypogonadism Delayed puberty Low testosterone level Growth failure	Wheelchair dependency Loss of ability of self-feeding Gastric distension Respiratory insufficiency Cardiomyopathy	25–30 years old
	BMD	2–20 years old					Variable: 4th or 5th decade of life
Adult Muscular Dystrophy	MD1	20–60 years old	Myotonia Muscle weakness	Obesity Insulin resistance Hyperinsulinemia Hypertriglyceridemia Glucose intolerance	Hypogonadism Oligospermia Low testosterone levels Reduced fertility Erectile dysfunction Testicular atrophy	Hypogammaglobulinemia Pneumonia Cardiac arrhythmias	Variable: 5th or 6th decade of life
	MD2	~48 years old					

In summary, in muscular dystrophies which have childhood age of onset, such as DMD and BMD, there are common and well known clinical characteristics and complications that lead the patient to death (Table 1). But also some common metabolic and endocrine alterations have been identified in both DMD and BMD. One of the most important metabolic aspects is nutritional status. Obesity and malnutrition have been identified in these dystrophinopathies. Furthermore, there are some biochemical aspects that have demonstrated an impaired glucose and insulin metabolism. Moreover, these patients present hypogonadism, which is important to emphasize that hypogonadism consequences are related to the age of onset. For instance, since hypogonadism is present in early infancy, these patients are going to present delayed puberty and growth failure (Table 1). However, since DMD/DMB patients have short life expectancy, secondary consequences of hypogonadism such as reduced fertility and oligospermia are less relevant.

3. Adult Muscular Dystrophies

Clinical features in muscular dystrophies also can appear in juvenile or adult age, and these phenotypes differ depending on mutation type [78]. Some muscular dystrophies in juvenile or adult age include distal myopathy, Miyoshi and Nonaka [79], inclusion body myositis (IBM) [80], facioscapulohumeral muscular dystrophy (FSHMD) [81], oculopharyngeal muscular dystrophy (OPMD) [82], distal myopathy [83], myotonic dystrophy (MD, type 1 or type 2) [84], and limb-girdle muscular dystrophy (LGMD 1B) [79]. However, we focused this paper on two last dystrophies.

3.1. Clinical Characteristics of Myotonic Dystrophy and LGMD. Myotonic dystrophy (MD) is the most common inherited neuromuscular disease in adults, with a global incidence of 1/8000 individuals [85]. Two types of MD exist:

type 1 (MD1) and type 2 (MD2) [85, 86]. MD1 is a chronic, slowly progressive, highly variable inherited multisystemic disease. MD1 results from an unstable (CTG) expansion in 3' UTR of the MD protein kinase gene (MDPK) at 19q13.3 locus [84]. MD2 is caused by an unstable expansion of a CCTG tetraplet repeat in intron 1 of the ZFN9 gene localized on chromosome 3q21.3 [84]. The phenotypes of MD1 and MD2 have a broad spectrum of clinical signs that include mainly myotonia and muscle weakness. The first neuromuscular symptoms appear during a wide age range (20–60 years) [86]. Other clinical signs of these dystrophies include cataracts prior to 50 years of age, cardiac conduction defects, endocrine changes, testicular atrophy, insulin resistance, and hypogammaglobulinemia [84]. In MD2, clinical features appear in adulthood (median age 48 years) in contrast to adult-onset MD1 and childhood onset [87]. The majority of patients (63%) die between 50 and 65 years of age [86, 87]. Pneumonia and cardiac arrhythmias are the most frequent primary causes of death, each occurring in 30% of patients, which was much higher than expected for the general population [88].

Other types of dystrophy are LGMD, which describes a heterogeneous group of muscle disorders characterized by a predominant proximal distribution of limb-girdle, shoulder, and hip weakness [79]. At least 15 different genetic forms of LGMD are now known [79]. The phenotypes begin during early childhood to late adulthood [89]. The LGMD group is still growing today and consists of 19 autosomal dominant and recessive forms (LGMD1A to LGMD1G and LGMD2A to LGMD2M) [84, 89]. The proteins involved are diverse and include sarcomeric, sarcolemmal, and enzymatic proteins [90].

3.2. Endocrine Alterations. Endocrine abnormalities have been reported in MD2 and MD1 patients (Figure 4), including the alteration in testicular function such as hypogonadism

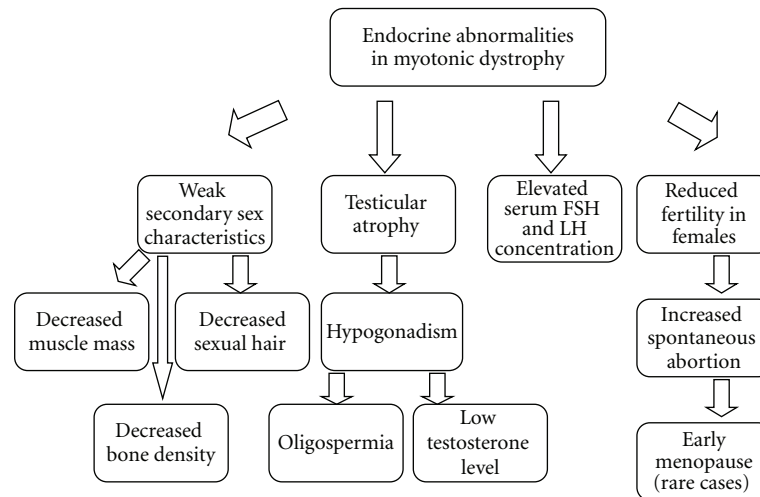


FIGURE 4: Endocrine abnormalities described in MD1/MD2.

leading to oligospermia [91], low levels of testosterone (T), and reduced spermatogenesis [92], resulting in reduced male fertility. In fact, hypogonadism may be a cause for erectile dysfunction (ED), which has been demonstrated in 25% of patients with MD1 [93]. The occurrence of ED is independent of the patient's age but may be related to other intrinsic factors of MD1 such as disease duration and severity and CTG expansion [94, 95]. Also, those patients present elevated levels of follicle-stimulating hormone (FSH) and reduced T level in comparison to control subjects [92, 93, 96].

Hypogonadism is very common in males with myopathies and involves both interstitial (androgenic) and tubular (spermatogenic) gonadal functions. In primary hypogonadism, luteinizing hormone (LH) increases and T level is reduced [93, 94]. In contrast, in compensated hypogonadism, LH increases and T levels are normal [93].

Testicular atrophy is reported to be the most prominent feature in ~80% of MD1 patients [97, 98]. Testicles of MD1 patients are characterized by an increase in the number and size of Leydig cells as well as tubular atrophy, hyalinization, fibrosis of the seminiferous tubules and reduced spermatogenesis. It has been reported that 46% of MD1 patients show hormonal evidence of interstitial gonadal failure [97, 99].

3.3. Metabolic Alterations

3.3.1. Malnutrition. Although MD patients demonstrate disorders of the oropharyngeal cavity, myotonia of the tongue and pharynx, impaired pharyngeal contraction, and slowing of esophageal peristalsis, to date, there have been no reports regarding the malnutrition state related to MD [4]. Loss of ability to cut and manipulate food leads to a loss of the ability of a person with MD to ensure adequate nutrition [100]. In this context it has been reported that 62% of patients with MD1 do not meet their daily energy requirements according to government recommendations: 55% of MD1 patients had a fat intake higher than the acceptable macronutrient distribution ranges [4]. Furthermore, 10% of the MD1 group of

patients were categorized as obese (BMI > 30) and 13% had BMI values <18.5, which is in the underweight category [4]. Patients with MD1 have macronutrient and energy intake deficiencies as well as an insufficient intake of minerals (copper, zinc, and calcium) [57]. However, there are no reports regarding malnutrition in MD patients.

3.3.2. Obesity. In muscular dystrophy, plasma membrane is damaged, generating myofibers passing through cycles of deterioration and regeneration until the end of its repair capacity [25, 28–30]. This induces the muscle fibers to be susceptible to the development of necrosis and to be replaced by fibrous connective tissue and adipose tissue [30]. Fibrotic tissue is still considered as lean tissue, so the increase in fat mass may be considered as a reflection of fat involution in muscles [30–32]. Myotonic dystrophy is linked to metabolic syndrome including insulin resistance, increased fat mass, and hypertriglyceridemia [58].

Progressive muscle loss associated with fat infiltration will carry a decrease in motor function and an increase in whole body fat mass index and regional fat-free mass index [55, 56]. Therefore, there is a progressive worsening of disease leading to a decrease of the vital capacity as well as total lung capacity and increases in fat mass [56]. MD1 patients present lower regional (legs, arms, and trunk), fat-free mass index (FFMI), and higher fat mass index (FMI) than healthy individuals [101]. In MD1 patients, a correlation has been reported with an increased total fat-free mass index and decreased motor function and with both decreasing vital capacity and total lung capacity [101, 102].

Aitkens et al. [103] observed that patients with neuromuscular disease showed more cases of obesity and were more sedentary than control subjects (37% versus 34%). However, in this study only 11 patients with neuromuscular disease were analyzed, and only four of the patients had myotonic dystrophy [103]. Therefore, this is not the best reference to determine the prevalence of obesity in MD patients [54]. Recently, Kaminsky et al. [102] studied 106 patients

with MD1 (46 males and 58 females) within a range of 55 years of age. These authors reported that the prevalence of obesity was 25.6% and hypertriglyceridemia was 47.6% [102]. The increased fat mass in MD1 patients could be considered a reflection of fat involution in muscles and a link with the metabolic disturbances in these patients [102].

3.3.3. Glucose and Insulin Metabolism. Patients with myotonic dystrophy have alterations in glucose metabolism, and it has been reported that these individuals have insulin resistance as an early manifestation [101]. Insulin resistance is the main cause of glucose intolerance in MD1 and, as a consequence, hyperinsulinemia may coexist such as a compensatory mechanism and may later lead to the onset of diabetes mellitus [101, 103].

However, the prevalence of diabetes mellitus in MD1 patients has not been proven. Muscle wasting and the low physical activity can make worse insulin resistance and lead to deregulation of protein catabolism [104].

Some hypotheses have been described to explain molecular insulin resistance in MD patients. One hypothesis is in regard to insulin receptor and the two existing isoforms: isoform A (IR-A), which lacks exon 11, and isoform B (IR-B), which includes exon 11 [105]. The insulin receptor B (IR-B) predominates in insulin-responsive tissues such as skeletal muscle. Interestingly, patients with MD1/MD2 express predominant insulin receptor isoform type A (IR-A) in skeletal muscle [106]. With a histological evaluation of MD muscle biopsies, it has been shown that the splicing changes in IR precede histological abnormalities [105]. The results showed that alterations in splicing occur prior to development of dystrophic changes, and this abnormal splicing may be a result of altered RNA binding due to the CUG expansion in the DMPK gene [101, 106].

Another metabolic alteration associated with glucose is abnormal insulin secretion in MD patients with normal insulin sensitivity [101]. This suggests damage to the β -cell secretory profile. This damage was represented by increased plasma proinsulin concentrations and a remarkably higher-than-normal early secretory response after oral glucose tolerance test in MD1 patients [107, 108]. A possible reason for this abnormal insulin secretion may be related with the protein kinase and the CUG expansion in the DMPK gene [108]. Protein kinase is involved in the modulation of the Ca^{+2} homeostasis in skeletal muscle cells, and Ca^{+2} homeostasis is crucial for β -cell secretion events [109]. If the alteration of calcium metabolism of the skeletal muscle also affects the β -cell, then the abnormal pattern of insulin secretion may be related to a malfunction of the MD1 protein kinase [109].

In summary, it has been reported that MD patients present obesity with a prevalence of 25.6% and that it is related to muscle atrophy. Obesity may be related to insulin resistance and metabolic disturbances. However, obesity does not explain damage in glucose metabolism because in myotonic individuals this metabolic alteration has been related to CUG expansion in the DMPK gene, which causes splicing changes in IR. This metabolic alteration has also been related to problems with insulin secretion. Therefore, all types of

damage in glucose metabolism in MD patients are related to the problem in the gene itself and not with the problems caused by the pathology. In this context, in DMD it has been noted that the metabolic problems are as a result of the pathology, for example, damage in membrane permeability, problems with glycolytic enzymes, lower glucose metabolism substrates, and changes in the sarcolemma.

On the other hand, it is important to denote that in adult muscular dystrophies such as MD, metabolic abnormalities are similar to those identified in childhood muscular dystrophies (Table 1). Additionally, in MD1 and MD2 some endocrine alterations have been identified (Table 1). One of these alterations is hypogonadism, which has also been identified in childhood muscular dystrophies (DMD/BMD). In this case, since onset is during adulthood, the primary and more relevant consequences of hypogonadism are related to reduced fertility, reduced spermatogenesis, low testosterone levels, and erectile dysfunction. In contrast to DMD/BMD, these patients had a normal growth and puberty.

4. Conclusions

Clinical and genetic characteristics of muscular dystrophies are diverse but all have one common characteristic: muscular atrophy. The muscle is one of the main tissues that regulates lipid and glucose metabolism by hormones such as insulin. It is important to consider that muscular dystrophies are related to weakness, fatigue, decreased mobility, and reduced physical working capacity. In addition to the muscular atrophy in these pathologies, replacement of skeletal muscle for fat and fibrotic tissue produces a reduction of the muscular mass; therefore, there is an imbalance for all these tissue functions. The combination of increased adiposity and sedentary lifestyle increases the risk for the development of metabolic syndrome. Age is an important factor in the muscular dystrophies, which produces the differences in clinical manifestation because age causes a more rapid progression of clinical, metabolic, and hormonal problems. For example, DMD patients often have short stature, whereas MD patients do not have height-related issues. DMD/BMD patients are frequently wheelchair-bound, and MD individuals do not present this limitation. DMD patients have problems with delayed puberty, whereas MD patients may have reproductive capabilities and have a functional sexual life. Metabolic problems may also increase if clinical manifestations begin in early age as in DMD and BMD. In this type of dystrophy, obesity is observed in the first decade, and during the course of time these patients may show malnutrition, whereas MD patients only develop obesity. Knowledge in regard to metabolic, physiological, and molecular alterations in muscular dystrophies will provide tools that will improve the quality of life for these patients.

Abbreviations

DMD:	Duchenne muscular dystrophy
BMD:	Becker muscular dystrophy
EDMD:	Emery-Dreifuss muscular dystrophy
DYS:	Dystrophin C-terminus

hGH:	Growth hormone
GC:	Glucocorticoids
REE:	Resting energy expenditure
BMI:	Body mass index
ST:	Skinfold thickness
DXA:	Dual energy X-ray absorptiometry
BIA:	Bioelectrical impedance analysis
MRI:	Magnetic resonance imaging
TMA:	Trimethyl amides
IBM:	Inclusion body myositis
FSHMD:	Facioscapulohumeral muscular dystrophy
OPMD:	Oculopharyngeal muscular dystrophy
MD:	Myotonic dystrophy
MDPK:	Protein kinase gene
LH:	Luteinizing hormone
FFMI:	Fat-free mass index
FMI:	Higher fat mass index
IR-A:	Isoform A-Insulin receptor
IR-B:	Isoform B-Insulin receptor.

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Research Article

Metabolic Context Regulates Distinct Hypothalamic Transcriptional Responses to Antiaging Interventions

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The hypothalamus is an essential relay in the neural circuitry underlying energy metabolism that needs to continually adapt to changes in the energetic environment. The neuroendocrine control of food intake and energy expenditure is associated with, and likely dependent upon, hypothalamic plasticity. Severe disturbances in energy metabolism, such as those that occur in obesity, are therefore likely to be associated with disruption of hypothalamic transcriptomic plasticity. In this paper, we investigated the effects of two well-characterized antiaging interventions, caloric restriction and voluntary wheel running, in two distinct physiological paradigms, that is, diabetic (*db/db*) and nondiabetic wild-type (*C57/Bl/6*) animals to investigate the contextual sensitivity of hypothalamic transcriptomic responses. We found that, both quantitatively and qualitatively, caloric restriction and physical exercise were associated with distinct transcriptional signatures that differed significantly between diabetic and non-diabetic mice. This suggests that challenges to metabolic homeostasis regulate distinct hypothalamic gene sets in diabetic and non-diabetic animals. A greater understanding of how genetic background contributes to hypothalamic response mechanisms could pave the way for the development of more nuanced therapeutics for the treatment of metabolic disorders that occur in diverse physiological backgrounds.

1. Introduction

The hypothalamus plays a pivotal role in mediating the central control of somatic energy metabolism and regulation of higher central nervous function throughout life. This ability of the hypothalamus to act as a bridge between central and peripheral systems has made it an important target for the study of age-related decline in metabolism and cognition [1–3]. Hypothalamic neuronal structure and function are dynamically regulated by homeostatic challenges, including feeding, fasting, and physical activity [4, 5]. In addition, genetic animal models with perturbations of somatic energy metabolism have also been shown to exhibit significant alterations in hypothalamic plasticity [6]. The neuroendocrine control of food intake and energy expenditure is associated

with, and likely dependent upon, hypothalamic synaptic plasticity. Reinforcing the importance of energy metabolism, both cellular or somatic, in homeostatic stability during lifespan, multiple studies have demonstrated the ability of experimental interventions that either curtail energy intake (caloric restriction) or increase energy expenditure (physical exercise) to ameliorate age-related pathophysiology [7–11]. Severe disturbances in energy metabolism, such as those that occur in obesity that significantly disrupt healthy aging for multiple reasons, are therefore likely to be associated with disruption of hypothalamic neuroplasticity. Maladaptive circuit alterations may also contribute to deleterious increases in hunger and reduced energy expenditure in the context of excessive adiposity. Mice possessing an inactivating mutation of the cognate cytokine receptor for leptin, *db/db* mice, are

obese, insulin resistant [12], and demonstrate an increased excitatory drive onto orexigenic neuropeptide Y-expressing hypothalamic neurons [13]. Leptin also contributes to the development of hypothalamic responsiveness and integration of food intake and metabolism [6] and also promotes morphological plasticity among hypothalamic neurons in the adult brain [14]. Various molecular transcriptomic signatures that respond to changes in energy intake and expenditure have now been characterized [15–18]. Consistent with leptin's role in energy metabolism, several leptin-responsive target genes have been identified in the hypothalamus [18, 19]. Challenges to metabolic homeostasis, such as caloric restriction (CR [20]) and voluntary wheel running [21, 22] are also associated with changes in hypothalamic gene transcription. Both reduced energy intake, through CR, and increased energy expenditure, through wheel running, have been associated with neuroendocrine alterations that are likely to be accompanied by dynamic alterations in hypothalamic neuroplasticity [4, 23]. However, a comprehensive picture of the transcriptional alterations that occur in both diabetic and nondiabetic animals in response to complex energetic challenges that prolong life and health span in multiple species, such as CR or wheel running, has yet to be determined. Such a question is important considering the multiple alterations in hormone levels, feedback responses and receptor functionality that occur in distinct physiological contexts, as these may disrupt the efficacy of the applied antiaging interventions. In order to assess global alterations in gene expression patterns following energetic challenges, we compared hypothalamic transcriptional profiles, following running or CR, in a diabetic context, that is, leptin receptor-deficient (*db/db*) mice compared to non-diabetic C57Bl/6 controls. Our study therefore is aimed at appreciating how the prevailing health context background can affect the applied therapeutic antiaging interventions of CR or exercise.

2. Materials and Methods

2.1. Animal Husbandry and Activity Monitoring. Animal care and experimental procedures followed NIH guidelines and were approved by the National Institute on Aging Animal Care and Use Committee (293-LNS-2010). For microarray analyses, male leptin receptor mutant (*db/db*, $n = 24$) mice, bred on a C57Bl/6 background, were purchased from The Jackson Laboratories. Age-matched male C57Bl/6 mice (wild type, $n = 24$) were used as controls. To validate the cycling conditions used to detect leptin mRNA in the brain, hippocampal tissue from ($n = 3$) *ob/ob* mice and ($n = 3$) wild-type mice was obtained and analyzed. Animals were one month old at the start of experiments. Mice from each genotype were kept in individual cages containing a running wheel equipped with an automated, computerized monitoring system. The running wheel was continuously available to the mice. The number of wheel rotations per day for each mouse was continuously recorded using MedSci Behavior monitoring software (Columbus Instruments, Columbus, OH). An additional cohort of ($n = 6$) C57Bl/6 male mice and ($n = 8$) *db/db* mice was used for *in situ* hybridization experiments, to confirm microarray

data. During the initial two weeks of the experiment, all mice were fed *ad libitum*, and food weights were recorded daily by experimenters. *Ad libitum* feeding levels were initially monitored for control and *db/db* animals, and then a diet that would supply sixty percent of their individual mean food intake was applied to generate forty percent caloric restriction (40% CR). This level of restricted feeding was chosen based on previous experiments [15, 16, 24]. The vivarium was maintained on a twelve hour light/dark cycle; all mice assigned to the CR diet were fed once daily at the onset of the dark period (18:00 hrs). Body weights were recorded on a weekly basis.

2.2. Hypothalamic Tissue Preparation. For *in situ* hybridization, mice were deeply anesthetized with Isoflurane, then perfused with 4% paraformaldehyde in phosphate buffer. Brains were postfixed in 4% paraformaldehyde with progressively increasing concentrations of sucrose, then stored at -80°C prior to sectioning. Hemibrains were sectioned at $40\ \mu\text{m}$ in the coronal plane using a freezing microtome (Microm M450, Fisher Scientific, Pittsburgh, PA). Sections were collected in a 1 : 6 series and stored in 4% paraformaldehyde. For microarray analysis and semiquantitative RT PCR, mice were anesthetized with isoflurane as described above, decapitated, and the brains were removed for hypothalamic dissection on ice. Dissected hypothalamic samples were frozen on dry ice and stored at -80°C prior to RNA extraction. Trunk blood was collected for serum analyses of metabolic and stress hormones, lipids; and ketone bodies and was stored at -80°C until used.

2.3. Circulating Metabolic Hormone and Lipid Measurements. For measurement of fasting glucose levels, food was removed from the cages of the mice on the *ad libitum* diet, and the daily allotment of food was withheld from mice on the 40% CR diet, the evening before glucose testing (17:00 hrs). The following morning, animals were briefly restrained, and glucose levels were measured following tail nick using a Therasense handheld analyzer (Therasense, Alameda, CA). Cholesterol and triglycerides were measured from trunk blood (after euthanization) using a Roche Cobas Fara II robotic chemical analyzer according to the manufacturer's specifications. All reagents for these analyses were purchased from Wako Diagnostics (Richmond, VA). Total cholesterol levels were determined using a kit (catalog no. 439-17501), as were triglyceride levels (catalog no. 461-08992). Insulin and leptin concentrations in serum samples were determined by ELISA (Crystal Chem., Inc., Downers Grove, IL). These assays were performed according to the manufacturer's instructions. Briefly, a microtiter plate coated with mouse antiinsulin or guinea pig antileptin antibody was washed three times with wash buffer (50 mM Tris-buffered saline (TBS) containing Tween 20). Five microliters of diluted standards and serum samples were added to wells in duplicate. Detection antibodies conjugated to the appropriate species were applied, and the plate was sealed and incubated for 2 hours while shaking. The wells were then washed and the enzyme solution was incubated for 30 minutes. After

washing, wells were reacted with substrate solution (o-phenylenediamine). Once the color developed sufficiently (15 minutes), stop solution (1 N sulfuric acid) was added, and the plate was read at 490 nm on an automatic plate reader (Perkin Elmer HTS 7000 Plus Bio Assay Reader, Perkin Elmer, Waltham, MA).

2.4. Circulating Corticosterone Measurements. Corticosterone levels were measured using a commercially available Radioimmunoassay (RIA) kit (Diagnostic Products Corp., Los Angeles, CA) according to the manufacturer's instructions. Serum was separated from trunk blood by centrifugation at 14,000 rpm for two minutes. Serum samples were stored at -80°C prior to analysis. Samples and corticosterone standards were thawed at room temperature and added to antibody-coated tubes in duplicate. 1.0 mL of (^{125}I -) labeled corticosterone was added, and each tube was vortexed before incubation for two hours at room temperature. Tubes were then decanted and counted using a Packard Cobra (5010) gamma counter.

2.5. Illumina Oligonucleotide Microarray. RNA isolation was carried out using the Qiagen RNeasy Mini Kit for animal tissues (Qiagen, Inc., Valencia, CA). In short, frozen tissues were cut into small pieces and allowed to thaw at 4°C in RLT lysis buffer (Qiagen). Tissue sections were disrupted using a Mini-Beadbeater-8 and 1.0 mm glass beads (BioSpec Inc., Bartlesville, OK). Tissue samples were then centrifuged, the supernatant transferred to a second tube and centrifuged again for cell debris clarification. The supernatant was added to 95% ethanol, mixed, and added to the binding columns. The columns were centrifuged, washed several times and the bound RNA was eluted using water. The RNA quality and quantity was checked using an Agilent 2100 bioanalyzer and RNA 6000 nano-chips. Total RNA was used to generate biotin-labeled cRNA using the Illumina TotalPrep RNA Amplification Kit (Ambion; Austin, TX). Briefly, $0.5\ \mu\text{g}$ of total RNA was first converted into single-stranded cDNA with reverse transcriptase using an oligo-dT primer containing the T7 RNA polymerase promoter site and then copied to produce double-stranded cDNA molecules. Double-stranded cDNA was used in an overnight *in vitro* transcription reaction where single-stranded RNA (cRNA) was generated and labeled by incorporation of biotin-16-UTP. A total of $0.75\ \mu\text{g}$ of biotin-labeled cRNA was hybridized at 58°C for 16 hours to Illumina's Sentrix MouseRef-8 Expression BeadChips (Illumina, San Diego, CA). Arrays were then washed, blocked and the labeled cRNA was detected by staining with streptavidin-Cy3. The arrays were scanned using an Illumina BeadStation 500X Genetic Analysis Systems scanner, and the image data extracted using the Illumina BeadStudio software, version 3.0.

2.6. Microarray Data Analysis. Microarray data were analyzed using DIANE 6.0, a spreadsheet-based microarray analysis program based on the SAS JMP7.0 system. Raw microarray data were subjected to filtering and Z-normalization and tested for significant changes as described previously [25]. Briefly, initial filtering identified genes with Z-ratio \geq

1.50, with the Z-ratio derived from the difference between the averages of the observed gene Z scores, divided by the standard deviation of all of the differences for that particular comparison. Genes were then refined by calculating the false discovery rate (FDR), which controls for the expected proportion of falsely rejected hypotheses, and including only those genes with $\text{FDR} < 0.05$. These data were further analyzed using a 2×3 ANOVA design with significance set at $P < 0.05$. The ANOVA design compared across genotype (*db/db* versus *C57Bl/6*) and environmental condition (sedentary/*ad libitum* versus sedentary/caloric restriction versus runner/*ad libitum*). This allowed us to identify transcripts that differed in their intensity across the various conditions in normal and leptin receptor-deficient mice. For functional genomic pathway analysis, specific significantly regulated gene lists were analyzed using Ingenuity version 8.6. The reference gene set was defined through the Ingenuity knowledge base (Genes Only). Inclusion in a pathway was defined as having a direct or indirect relationship with that pathway. For each pathway identified, the inclusion of at least two genes was required from the input dataset with a probability value of < 0.05 . The resultant data were also filtered by considering only those molecules and relationships, where species = mouse, and tissue = nervous system.

2.7. In Situ Hybridization. Generation of riboprobes for *in situ* hybridization was performed as described previously [26]. Probe sequences were as follows: for *Pias2*, right primer agtgttactgggctttgctg, left primer tctcaaaaggtgggcttagtg; for *Slc17a6*, right primer tggcacatgcatcctacag, left primer ccctccctttacaagctctc. Target genes were selected on the basis of significance derived from the microarray analysis, and on selective expression enrichment in the hypothalamus based on interrogation of the Allen Brain Atlas [27]. Leptin primer sequences were as follows: right primer tgtccaagatggaccagactc, left primer actggtctgaggcaggagca. Animal tissue for *in situ* hybridization was processed in separate runs that were balanced to include equal numbers of animals from each experimental condition. *In situ* hybridization was carried out as described previously [26]. Densitometric analysis was performed upon scanned images generated using a Typhoon Phosphorimager (GE Healthcare, Piscataway, NJ). Regions of interest from matched sections were user defined with ImageQuant (GE Healthcare) by an experimenter blind to the specific experimental group conditions. Five bilateral sections were averaged to obtain a single score for each animal. In order to validate our microarray studies, which measured gene expression changes across the whole hypothalamus, we sampled across all hypothalamic nuclei for *in situ* hybridization analyses. Sampling extended from the lateral preoptic area rostrally (Bregma +0.14 mm) to the lateral hypothalamic area caudally (Bregma -2.80 mm) according to the atlas of Paxinos and Franklin [28]. Anatomical sampling was balanced across groups to include one section containing the lateral preoptic area (Bregma +0.14 mm to -0.22 mm); one section spanning the anterior hypothalamic area, medial preoptic nucleus, suprachiasmatic nucleus, paraventricular nucleus, and lateral hypothalamus (Bregma -0.34 mm to Bregma -1.06 mm); one section

containing paraventricular nucleus, ventromedial hypothalamus, arcuate nucleus, dorsomedial hypothalamic nucleus, and lateral hypothalamus (Bregma -1.22 mm to Bregma -1.70 mm); one section containing the posterior hypothalamic area, lateral hypothalamus, dorsomedial hypothalamus, ventromedial hypothalamus, and arcuate nucleus (Bregma -1.82 mm to Bregma -2.30 mm); one section containing the posterior hypothalamic area, lateral hypothalamus, and arcuate nucleus (Bregma -2.46 mm to Bregma -2.80 mm).

2.8. Semiquantitative Real-Time PCR. Primer sequences and expected product sizes are shown in Table S1 Supplementary Material available online at doi: 10.1155/2012/732975. For genes with multiple transcript variants (IGF1 and NTRK3), Biology Workbench (version 3.2) was used to align the sequences, and primers were generated based on the consensus sequence. RNA sample quality was assessed through gel electrophoresis and spectrophotometric analysis. Total RNA samples were also treated with $1.0 \mu\text{L}$ DNase I ($2 \text{ U}/\mu\text{L}$; Ambion, Austin, TX) to remove any genomic DNA. RNA samples ($0.5 \mu\text{g}$) were converted to cDNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) with oligo-dT and random hexamers. Samples were also run in the absence of SuperScript III to evaluate the potential for genomic DNA contamination. cDNA samples ($4.0 \mu\text{L}$) were added to a $50 \mu\text{L}$ PCR amplification using Platinum PCR Supermix (Invitrogen, Carlsbad, CA), with gene-specific primers (Table S1). The linear range of PCR amplification for each primer set was determined in pilot experiments (25–35 cycles). Cycling conditions were a single 5-minute step at 95°C , followed by the appropriate number of amplification cycles, with annealing at 58°C . For leptin detection, PCR products were verified with DdeI restriction enzyme digest as described on The Jackson Laboratories website (<http://jaxmice.jax.org/protocols>). PCR products were run in a 1.2% agarose gel containing $0.5 \mu\text{g}/\text{mL}$ ethidium bromide at 100 V for one hour. Images were acquired under ultraviolet light using a BioRad ChemiDoc molecular imaging system (BioRad, Hercules, CA). Band intensities were quantified using NIH ImageJ (<http://rsbweb.nih.gov/ij/>) and expressed relative to the band intensity for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.9. Statistical Analysis. *In situ* hybridization and semiquantitative PCR data were compared across genotypes using bidirectional Student's *t*-tests (GraphPad Prism v.5, La Jolla, CA). Endocrine data were analyzed using one-way ANOVA with planned *post hoc* comparisons (sedentary C57Bl/6 mice on the *ad libitum* diet compared to all other groups; sedentary *db/db* mice on the *ad libitum* diet compared to *db/db* runners; sedentary *db/db* mice on the *ad libitum* diet compared to *db/db* mice on CR). The same *post hoc* planned comparisons were applied to the data on body weight gain and food intake following 2×3 repeated measures ANOVA using SPSS version 18. For all analyses, statistical significance was set at $P < 0.05$.

3. Results

3.1. Body Weight and Food Intake Alterations following Energetic Challenges. In agreement with previous reports, the CR paradigm reduced body weight in both C57Bl/6 (WT) and *db/db* mice (Figure 1(a); $F_{5,35} = 16.84$, $P = 0.0001$) [29, 30]. In *db/db* mice, but not WT mice, voluntary wheel running also reduced body weight gain ($F_{5,35} = 2.94$, $P = 0.02$). Effects on body weight occurred in the context of attenuated hyperphagia in *db/db* runners ($F_{5,35} = 3.71$, $P = 0.01$; Figure 1(b)), without any change in food intake in WT runners.

3.2. Alterations in Metabolic Hormone, Stress Hormone, and Lipid Profiles following Running or Caloric Restriction. Fasting glucose levels were significantly reduced following CR in *db/db* mice (Figure 2(a); $F_{5,34} = 45.51$, $P = 0.0001$). In WT mice, CR lowered circulating insulin levels, and in *db/db* mice, both running and CR significantly attenuated hyperinsulinemia (Figure 2(b); $F_{5,42} = 4.65$, $P = 0.002$). A strong trend towards reduced total cholesterol in runners of both genotypes was also evident (Figure 2(c); $F_{5,31} = 2.48$, $P = 0.057$). The significantly elevated serum triglycerides in *db/db* mice (compared to WT) were significantly attenuated following running or CR (Figure 2(d); $F_{5,31} = 24.54$, $P = 0.0001$). Additionally, *db/db* mice demonstrated persistently elevated leptin levels, relative to WT mice (Figure 2(e); $F_{5,29} = 4.61$, $P = 0.004$). In agreement with previous studies performed in the Zucker rat model [31], running reversed the elevated corticosterone levels observed in *db/db* mice (Figure 2(f); $F_{5,30} = 11.81$, $P = 0.0001$).

3.3. Differential Hypothalamic Gene Expression Patterns in *db/db* Mice, Compared to WT Mice, under *Ad Libitum*, Sedentary Conditions. To establish a baseline comparison for hypothalamic gene transcripts that differ significantly in their expression between *db/db* and WT mice under *ad libitum* feeding (simplified to “*ad libitum*”) and sedentary (nonrunning) conditions, we assessed global hypothalamic gene expression using an Illumina bead microarray. 244 genes differed significantly in their expression between sedentary *db/db* and WT mice, the majority of which were upregulated (185 genes upregulated; Figure 3(a), Table S2). Among upregulated genes, transcriptional regulators were prominently represented, for example, eukaryotic translation initiation factor 3, subunit 1 (Eif3s1), coiled-coil domain containing 94 (Ccdc94), and DEAH (Asp-Glu-Ala-His) box polypeptide 9 (Dhx9). Among significantly downregulated transcripts (in *db/db* compared to WT), a strong representation of enzymes, membrane proteins, and mitochondrial-related gene transcripts was evident, for example, ectonucleotide pyrophosphatase/phosphodiesterase 5 (Enpp5), HLA-B-associated transcript 5 (Bat5), and translocase of outer mitochondrial membrane 22 (Tom22). Statistical signaling pathway analysis (Ingenuity Pathway Analysis: IPA) of the gene transcripts differentially expressed between the *db/db* and WT mice under *ad libitum*, sedentary conditions were linked primarily with “nervous system development,” “neurological disease,” and “molecular transport”

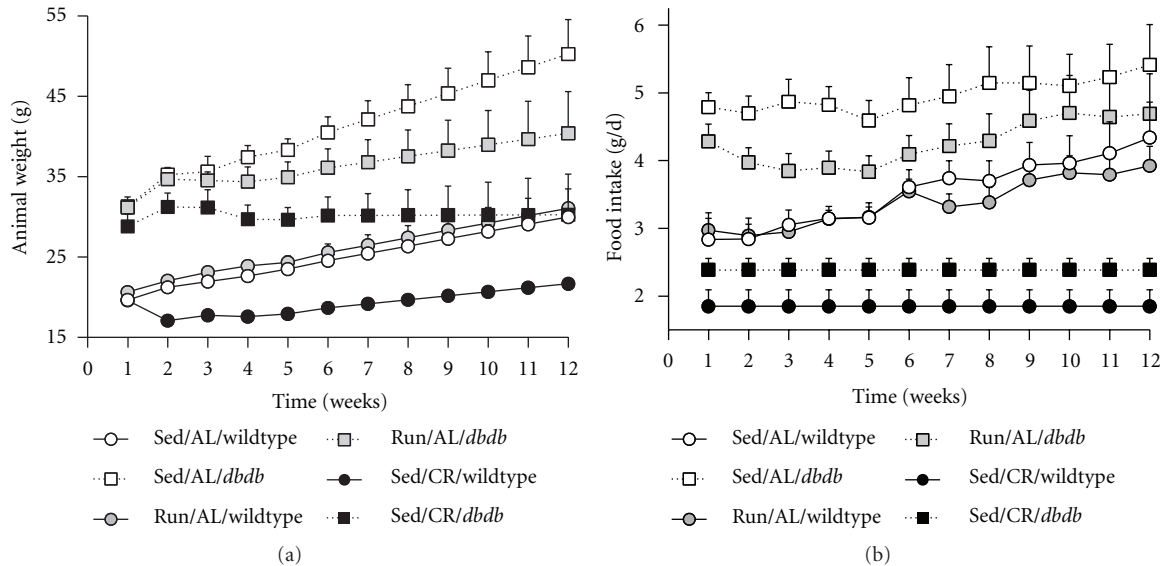


FIGURE 1: Caloric restriction and wheel running alter body weight gain and food intake in C57Bl/6 and leptin receptor-deficient mice. (a) C57Bl/6 mice maintained on 40% caloric restriction (CR) gain weight more slowly than wild-type mice on the *ad libitum* (AL) diet. *db/db* mice maintained on CR from one month of age did not significantly gain weight over the subsequent twelve weeks of the experiment, and voluntary wheel running decelerates body weight gain in *db/db* mice. (b) Running transiently suppresses food intake in *db/db* mice. Data were analyzed with 2×3 repeated measures ANOVA with Tukey's *post hoc* and significance set at $P < 0.05$. Error bars represent SEM.

(Figure 3(b)). The specific genes associated with the regulation of these highest probability-scoring signaling pathways included amyloid beta precursor-like protein 2 (*Aplp2*), cyclin-dependent kinase inhibitor 1B (*Cdkn1b*), amyloid precursor protein (*App*), and metabotropic glutamate receptor type 7 (*Grm7*). Additionally functional gene networks, created using IPA algorithms, from this dataset included pathways linked to “cell-to-cell signaling,” as well as “tissue and organ morphology.”

To validate multiple aspects of our hypothalamic microarray analysis, we characterized *db/db*-related differences in gene expression using *in situ* hybridization and semiquantitative reverse transcriptase PCR (RT-PCR). We measured the expression of four chosen transcripts differentially expressed in *db/db* mice compared to WT. From our array data both protein inhibitor of activated STAT2 (*Pias2*) and solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter) member 6 (*Slc17a6*) were significantly upregulated in the hypothalamus of *db/db* mice compared to WT. *In situ* hybridization of these genes confirmed their expression potentiation in *db/db* mice (Figures 3(c) and 3(d)). With RT-PCR, expression of glutathione peroxidase 7 (*Gpx7*) was elevated, and the expression of translocase of outer mitochondrial membrane 22 (*Tomm22*) was found to be reduced, both of which were consistent with the microarray results (Table S2).

3.4. Regulation of Hypothalamic Transcription Patterns by Running Activity in WT Mice. Experimental mice often voluntarily run long distances with an available running wheel. The WT mice employed in this study ran 6.68 ± 1.45 km/24 hr. When comparing the gene transcripts differentially regulated by running in WT mice compared to

their WT sedentary counterparts, we found that the majority of significantly altered genes were downregulated (~58%; Figure 4(a); Table S3) in response to running. This profound increase in activity potentiated the expression of transcripts involved with neuronal development and differentiation (FK506 binding protein 5, *Fkbp5*: [32]; SH3-domain GRB2-like 1, *Sh3gl1*: [33]; secreted frizzled-related protein 1, *Sfrp1*: [34]) and chromatin remodelling (vacuolar protein sorting 72, *Vps72*: [35]). Running activity, in contrast, depressed hypothalamic expression of transcripts related to appetite and metabolism (hypocretin/orexin, *Hcrt*: [36]; lipocalin 2, *Lcn2* [37]; leptin, *Lep* [38]) as well as insulin receptor signaling (tripartite motif-containing 72, *Trim72* [39]). Alterations in *Sfrp1* and *Fkbp5* and mRNA expression were confirmed through RT-PCR (Figures 4(b), 4(c)). Both PCR and *in situ* hybridization were used to detect leptin mRNA in the hypothalamus (Figure 4(d)). Hypothalamic leptin mRNA expression was detectable in sedentary mice using *in situ* hybridization (Figure 4(d)), consistent with previous reports demonstrating endogenous leptin mRNA expression in the hypothalamus [29, 30]. We compared hypothalamic leptin mRNA in hypothalamus with mRNA in white adipose tissue (WAT). Leptin mRNA expression was lower in the hypothalamus than WAT and as a control was absent in *ob/ob* hypothalamus (Figure 4(d)). Confirming our array data we found, with RT-PCR, that indeed hypothalamic leptin mRNA was reduced with running (Figure 4(d)). Investigating the signaling pathways populated by the differential running versus sedentary gene-set in WT mice, a strong neurodevelopmental phenotype was observed (Figure 4(b)). Running influenced the expression of numerous genes associated with “nervous system development,” “molecular transport,” and “cell morphology.” Genes

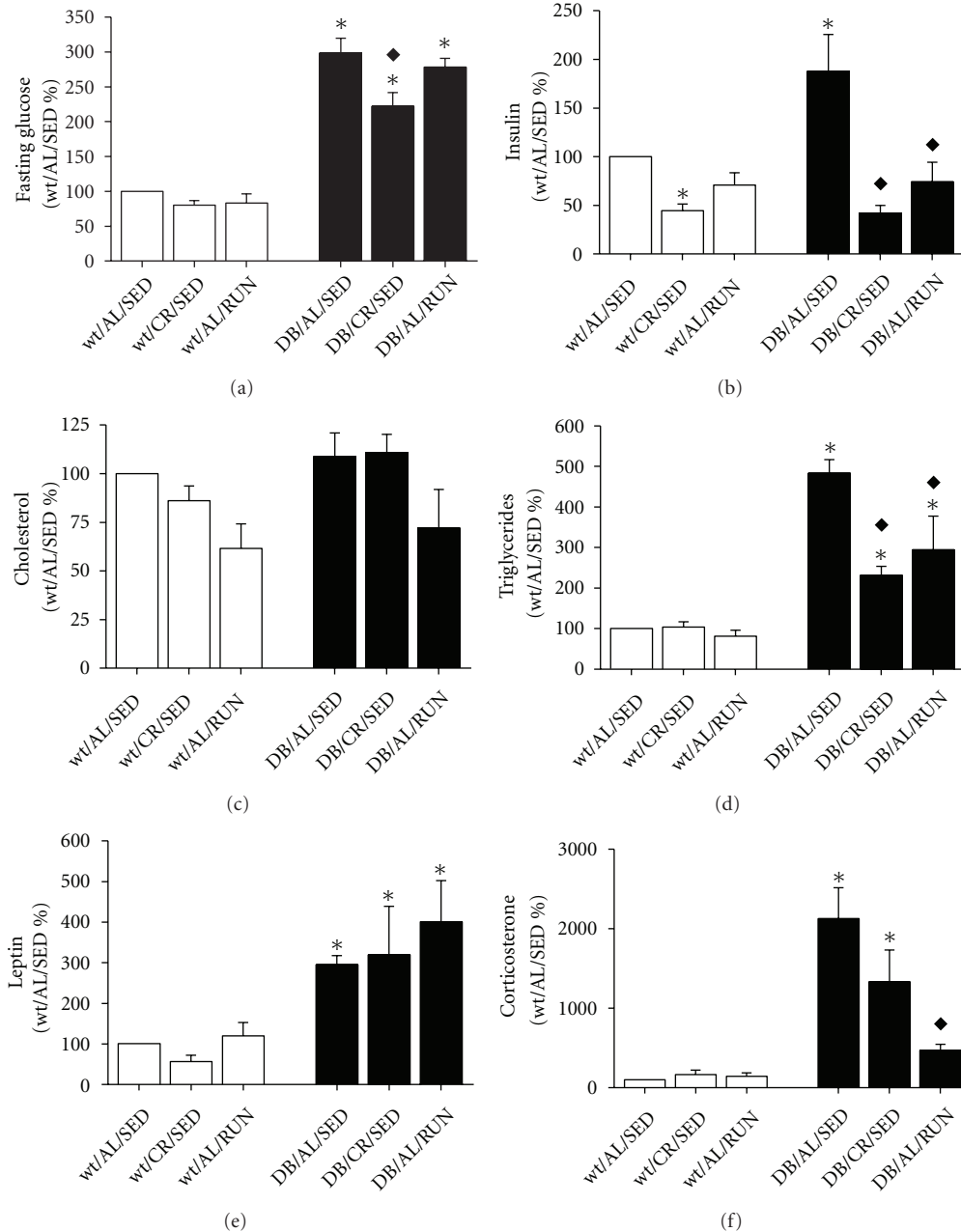


FIGURE 2: Endocrine changes in C57Bl/6 and *db/db* mice following running or caloric restriction. For all graphs, asterisk (*) indicates significance at $P < 0.05$ relative to sedentary C57Bl/6 mice fed *ad libitum*. Black diamonds (◆) represent significance at $P < 0.05$ relative to sedentary *db/db* mice fed *ad libitum*. (a) Caloric restriction (CR) attenuates fasting hyperglycemia in *db/db* mice. (b) CR lowers circulating insulin concentrations in C57Bl/6 mice, and both running and CR reinstate normal insulin levels in *db/db* mice. (c) Although there is a trend towards reduced total cholesterol in runners, this did not reach statistical significance. (d) Serum triglycerides are elevated in *db/db* mice, but this elevation can be ameliorated following running or CR. (e) Serum leptin levels are increased in *db/db* mice across all conditions. (f) Running attenuates the elevated corticosterone levels observed in *db/db* mice. Abbreviations: wt = C57Bl/6; AL = *ad libitum*; CR = caloric restriction; RUN = wheel running; DB = *db/db* mice.

implicated in nervous system development and function included leptin and the murine homolog of *Drosophila* Slit1. The gene encoding POU domain, class 3, transcription factor 3 (Pou3f3), was also represented in this category, as was the presynaptic modulator chordin (Chrd [40]). Serotonin receptor 1b (Htr1b), Sfrp1, NK2 homeobox 1 (Nkx2-1), and the progesterone receptor (Pgr) were also included as

components of the “nervous system development” pathway regulated by running in WT mice.

3.5. Regulation of Hypothalamic Transcription Patterns by Caloric Restriction in WT Mice. Following CR implementation in WT mice, 521 genes were significantly altered in the hypothalamus compared to *ad libitum*-fed WT

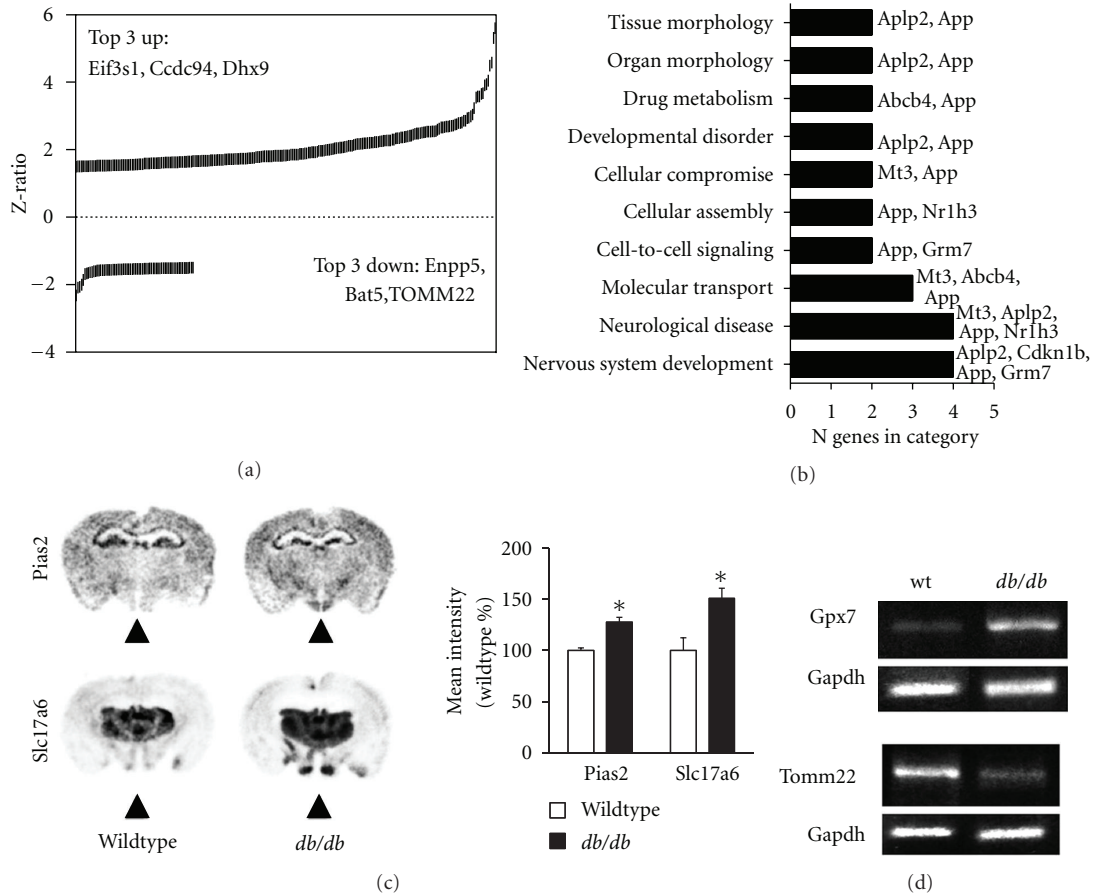


FIGURE 3: Differences in hypothalamic gene expression and pathway recruitment between C57Bl/6 and *db/db* mice under ad libitum, sedentary conditions. (a) Graph of Z-ratios showing that differences in gene expression between C57Bl/6 and *db/db* mice are primarily attributable to transcriptional upregulation, as opposed to down-regulation. Abbreviations: Eif3s1, eukaryotic translation initiation factor 3, subunit J; Ccdc94, coiled-coil domain containing 94; Dhx9, DEAH (Asp-Glu-Ala-His) box polypeptide 9; Enpp5, ectonucleotide pyrophosphatase/phosphodiesterase 5; Bat5, HLA-B associated transcript 5; Tomm22, translocase of outer mitochondrial membrane 22. (b) Pathways that differ between wild-type and *db/db* mice under *ad libitum*, sedentary conditions. Aplp2, amyloid beta (A4) precursor-like protein 2; App, amyloid beta precursor protein; Abcb4, ATP-binding cassette, subfamily B member 4; Mt3, metallothionein 3; Nr1h3, nuclear receptor subfamily 1, group H, member 3; Grm7, glutamate receptor, metabotropic 7; Cdkn1b, cyclin-dependent kinase inhibitor 1B. (c) Pias2 and Slc17a6 expression in C57Bl/6 and *db/db* mice under *ad libitum*, sedentary conditions. Arrowheads indicate the hypothalamic nuclei, which were traced over the rostrocaudal extent of the hypothalamus to generate an average index of gene expression that would be directly comparable to the microarray analysis conducted in whole-hypothalamus RNA extracts. Pias2, protein inhibitor of activated STAT 2; Slc17a6, solute carrier family 17 member 6. Associated graph indicating densitometric results of the expression analysis. Consistent with the microarray results, both Pias2 and Slc17a6 were significantly upregulated in the hypothalamus of *db/db* mice. Asterisk (*) indicates statistical significance at $P < 0.05$ following bidirectional student's *t*-tests. (d) Further validation through semiquantitative PCR reveals that, as shown by the microarray, Gpx7 mRNA is increased and Tomm22 mRNA is reduced in the hypothalamus of *db/db* mice.

controls. Similar numbers of genes were significantly up- or downregulated (269 up-regulated, 225 downregulated; Figure 5(a), Table S4). Many of the upregulated transcripts are involved in regulating neurotransmission (otoflerin, Otof [41]; Wiskott-Aldrich syndrome protein interacting protein family 1, Wipf1 [42]; neurotrophin receptor tyrosine kinase 3, Ntrk3 [43]; calcium/calmodulin kinase 1D, CamK1d [44]) and differentiation YLP motif containing 1 (Ylpm1 [45]). CR in the WT animals, however, caused profound suppression of multiple energy-modulatory factors including insulin-like growth factor 1 (Igf1), phospholipid transfer protein (Pltp), and neuronal ceroid lipofuscinosis 6 (Cln6), which

were significantly downregulated following CR. Changes in Igf1, Ntrk3, and CamK1d were further validated by RT-PCR (Figures 5(b), 5(c)). Band intensities for each of the validated genes confirmed the differences shown by the microarray results.

The functional pathways that were significantly altered in the C57Bl/6 mice following CR included "neurological disease," "cell death," and "cellular growth and proliferation" (Figure 5(b)). Ntrk3, adenylate cyclase activating polypeptide 1 receptor 1 (Adcyap1r1), E2F transcription factor 1 (E2f1) and X-ray repair in Chinese hamster cells 6 (Xrcc6) were represented within the "neurological disease"

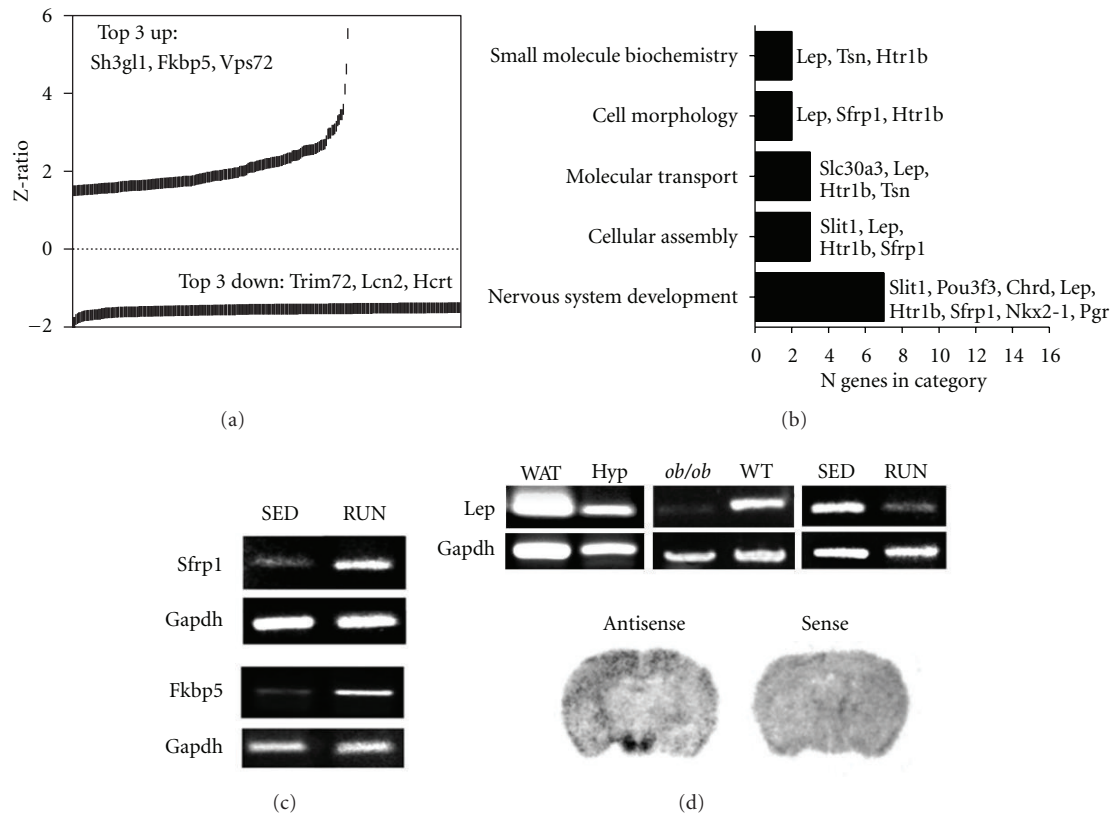


FIGURE 4: Wheel running influences hypothalamic gene expression and pathway activation in C57Bl/6 mice. (a) Z-ratios for gene transcripts up- or downregulated following voluntary wheel running in C57Bl/6 mice. Fkbp5, FK506 binding protein 5; Vps72, vacuolar protein sorting 72; Sh3gl1, SH3-domain GRB2-like 1; Lcn2, lipocalin 2; Hcrt, hypocretin; Trim72, tripartite motif-containing 72. (b), Pathways responsive to running in the hypothalamus of C57Bl/6 mice. Abbreviations: Lep, leptin; Htr1b, 5-hydroxytryptamine receptor 1B; Tsn, translin; Sfrp1, secreted frizzled-related protein 1; Slc30a3, solute carrier family 30, member 3; Slit1, slit homolog 1; Pou3f3, POU domain, class 3, transcription factor 3; Chrd, chordin; Nkx2-1, NK2 homeobox 1; Pgr, progesterone receptor. (c), PCR validation of running-induced transcriptional alterations in the hypothalamus of wild-type mice. (d) Leptin mRNA was detected in the hypothalamus samples using microarray, PCR, and *in situ* hybridization techniques. White adipose tissue (WAT) expresses leptin mRNA at higher levels than hypothalamus (Hyp) by PCR; *ob/ob* mice lack leptin bands at 155 bp in the hypothalamus following restriction enzyme digest, while wild-type (wt) controls express leptin mRNA. As shown by the microarray, running reduced endogenous leptin mRNA expression in the hypothalamus. *In situ* hybridization techniques were also used to detect endogenous leptin mRNA expression in the hypothalamus of sedentary wild-type mice.

pathway. Reinforcing its pluripotent role in both endocrine and neuronal health status, Igf1 was represented across multiple pathways, including “nervous system development”, “cell growth and proliferation”, and “neurological disease” (Figure 5(b)).

3.6. Regulation of Hypothalamic Transcription Patterns by Running Activity in *db/db* Mice. Diabetic *db/db* mice ran considerably less than their WT counterparts (0.33 ± 0.08 km/24 hr). Consistent with this reduced drive for voluntary activity, considerably fewer hypothalamic genes (compared to WT mice) were differentially expressed following running in *db/db* mice. 240 genes met our criteria for differential expression following running (compared to sedentary *db/db* mice) in the *db/db* mouse hypothalamus. Of those genes, the majority (~89%) were upregulated (Figure 6(a); Table S5). Significantly upregulated genes

included transcripts associated with neuronal protein modification involving lysosomal or ubiquitin function (N-acetylgalactosaminidase alpha, Naga [46]: HECT domain and ankyrin repeat containing, Hace1 [47]), and cell growth and cycle progression (neuregulin, Nrg1 [48]: HEAT repeat containing 5A, Heatr5a [49]). Among the down-regulated transcripts several are prominent controllers of neuronal excitability and development (glycine receptor alpha 1, Glra1 [50]: T-box brain gene 1, Tbr1 [51]), receptor expression stabilization (sorting nexin 1, Snx1 [52]), and pentose-phosphate metabolism (ribulose-5-phosphate-3-epimerase, Rpe [53]). Changes in Naga, Tbr1 and Nrg1 were further confirmed by RT-PCR (Figures 6 (b), 6 (c)). Band intensities for each of these genes were compared across *db/db* mice that ran or were sedentary, and the differences in band intensities were in accordance with the microarray results.

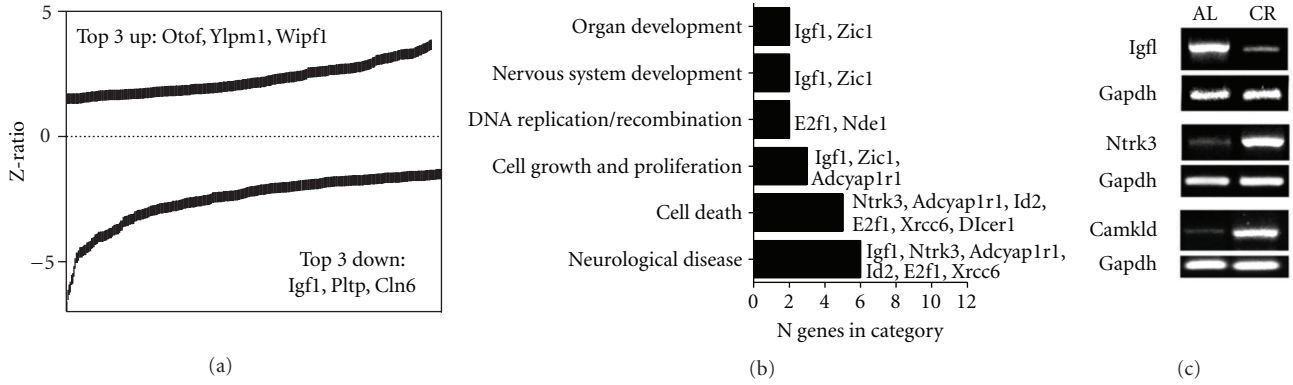


FIGURE 5: CR influences hypothalamic gene expression and pathway recruitment in wild-type mice. (a) Z-ratios for genes that were up- or down-regulated following CR in wild-type mice. Otof, otoferlin; Ylpm1, YLP motif containing 1; Wipf1, WAS/WASL interacting protein family, member 1; Igf1, insulin-like growth factor 1; Pltp, phospholipid transfer protein; Cln6, ceroid-lipofuscinosis, neuronal 6. (b) Pathway analysis of genes responsive to CR in wild-type mice. Abbreviations: Zic1, zinc finger protein of the cerebellum 1; E2f1, E2F transcription factor 1; Nde1, nuclear distribution gene E homolog 1; Adcyap1r1, adenylate cyclase activating polypeptide 1 receptor 1; Ntrk3, neurotrophic tyrosine kinase, receptor, type 3; Id2, inhibitor of DNA binding 2; Xrcc6, X-ray repair in Chinese hamster cells 6; Dicer1, Dicer1 homolog. (c) PCR validation of changes in gene expression following CR in wild-type mice.

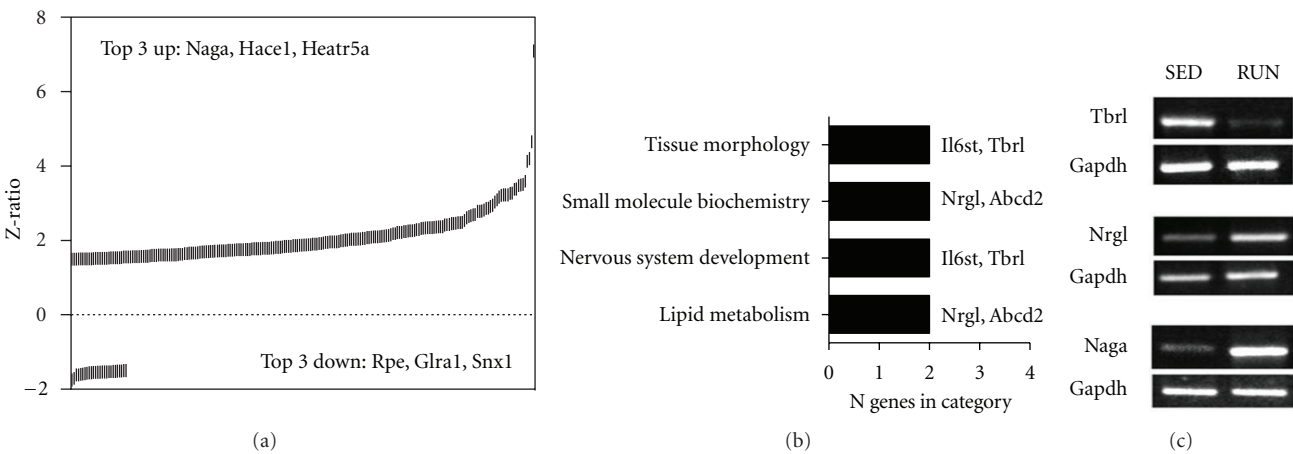


FIGURE 6: *db/db* mice respond to running with a distinct transcriptional profile. (a) Leptin receptor-deficient *db/db* mice exhibit transcriptional upregulation, indicated by positive Z-ratios, following twelve weeks of voluntary wheel running. Rpe, ribulose-5-phosphate-3-epimerase; Glra1, glycine receptor, alpha 1 subunit; Snx1, sorting nexin 1; Naga, N-acetyl galactosaminidase, alpha; Hace1, HECT domain and ankyrin repeat containing, E3 ubiquitin protein ligase 1; Heatr5a, HEAT repeat containing 5A. (b) Pathway analysis of genes regulated by voluntary exercise in *db/db* mice. Abbreviations: Il6st, interleukin 6 signal transducer; Tbr1, T-box brain gene 1; Nrg1, neuregulin 1; Abcd2, ATP-binding cassette, sub-family D, member 2. (c) PCR validation of differences in gene expression following running in *db/db* mice.

Functional gene pathway analysis of the genes significantly altered in *db/db* mice after running, revealed that four major functional categories, “nervous system development,” “small molecule biochemistry,” “tissue morphology,” and “lipid metabolism” were significantly represented (Figure 6(b)). Two genes associated with “lipid metabolism” and “small molecule biochemistry,” ATP-binding cassette, sub-family D, member 2 (Abcd2) and Nrg1 were influenced by running in *db/db* mice. Two genes implicated in “nervous system development and function” and “tissue morphology,” T-box brain gene 1 (Tbr1) and interleukin 6 signal transducer (Il6st), were also differentially regulated following running in *db/db* mice.

3.7. Regulation of Hypothalamic Transcription Patterns by Caloric Restriction in *db/db* Mice. Quantitatively, the functional effects of CR implementation on hypothalamic gene transcription were more conserved across both metabolic context, that is, WT to *db/db*, than were the effects of running. *db/db* mice exhibited statistically significant changes in 488 genes following CR, and the majority ($n = 287$ transcripts) were significantly down-regulated (Figure 7(a), Table S6). Among the down-regulated genes were factors linked to transmembrane receptor (kinesin family member 3A, Kif3a [54]) and neuronal ion channel activity (voltage-gated, type I, alpha subunit, Scn1a [55]; glutamic acid decarboxylase 1, Gad1). Genes up-regulated following CR

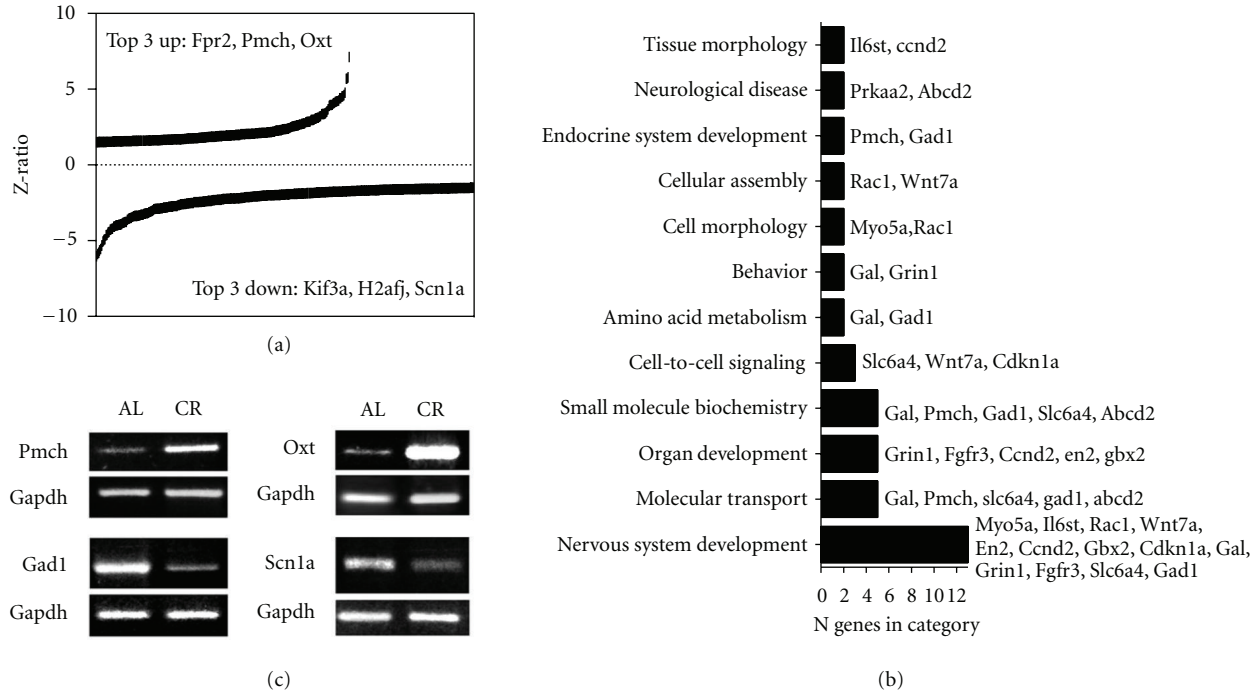


FIGURE 7: CR activates a diverse set of transcripts and pathways in *db/db* mice. (a) Z-ratios for genes that were up- or downregulated following CR in *db/db* mice. Fpr2, formyl peptide receptor 2; Pmch, pro-melanin-concentrating hormone; Oxt, oxytocin; Kif2a, kinesin family member 2A; H2afj, H2A histone family, member J; Scn1a, sodium channel, voltage-gated, type I, alpha. (b) Pathway analysis of genes responsive to restricted feeding in leptin receptor-deficient mice. Abbreviations: Il6st, interleukin 6 signal transducer; Ccnd2, cyclin D2; Prkaa2, protein kinase, AMP activated, alpha 2 catalytic subunit; Abcd2, ATP-binding cassette, subfamily D member 2; Pmch, pro-melanin-concentrating hormone; Gad1, glutamic acid decarboxylase 1; Rac1, RAS-related C3 botulinum substrate 1; Wnt7a, wingless-related MMTV integration site 7A; Myo5a, myosin VA; Gal, galanin; Grin1, glutamate receptor, ionotropic, NMDA1; Slc6a4, solute carrier family 6 member 4; Cdkn1a, cyclin-dependent kinase inhibitor 1A; Fgfr3, fibroblast growth factor receptor 3; En2, engrailed 2; Gbx2, gastrulation brain homeobox 2. (c) PCR validation of differences in gene expression after CR in *db/db* mice.

in *db/db* mice included transcripts for receptors and ligands associated with chemosensation and neuronal control of energy regulation (formyl peptide receptor 2, Fpr2 [56]; oxytocin, Oxt; pro-melanin-concentrating hormone, Pmch [57]). The expressions of Pmch, Oxt, Scn1a, and Gad1 were further evaluated using RT-PCR (Figures 7(b), 7(c)). Pmch and Oxt were increased, and GAD1 and Scn1a were decreased in these assays, in accordance with the microarray data.

Significantly populated signaling pathways recruited following CR in *db/db* mice included “nervous system development,” “neurological disease,” and “cell-to-cell signaling” (Figure 7(b)). Gad1, wingless-related integration site 7A (Wnt7a), and cyclin-dependent kinase inhibitor 1A (Cdkn1) were represented in the “nervous system development” functional network. The significantly-populated signaling pathway “neurological disease” comprised both protein kinase, AMP-activated, alpha 2 catalytic subunit (Prkaa2) and ATP-binding cassette, subfamily D, member 2 (Abcd2).

3.8. Common and Distinct Functional Pathways Alterations in Response to Running and Caloric Restriction in C57Bl/6 and *db/db* Mice. Under sedentary conditions with *ad libitum* feeding, *db/db* mice differed from C57Bl/6 mice in a

manner that was primarily attributable to transcriptional upregulation (Figure 8(a)). Numerically, there was a greater overlap between genotypes when comparing gene transcription following running (Figure 8(b)), relative to comparison across genotypes following CR (Figure 8(c)). Wheel running was associated with greater numbers of genes that were upregulated in *db/db* mice, while, in contrast, CR was predominantly associated with higher numbers of genes that were down-regulated in *db/db* mice (Figure 8(d)). 31 distinct, significantly populated, pathways were represented across the entire dataset. Among these pathways, only two categories contained genes that were significantly regulated across all experimental manipulations. These two categories were “nervous system development” and “tissue morphology”, suggesting that they are highly conserved functions.

To assess directly the ability of the two imposed antiaging paradigms (CR and running) to modulate hypothalamic transcription in the diverse genomic backgrounds (WT, *db/db*), we created Venn diagrams of the specific transcriptomic effects. We found that between the WT and *db/db* backgrounds, running appeared to regulate more common genes (302: Figure 9(a), Table S7) compared to CR (127: Figure 9(b), Table S8). However, despite the greater numerical conservancy of running effects (compared to

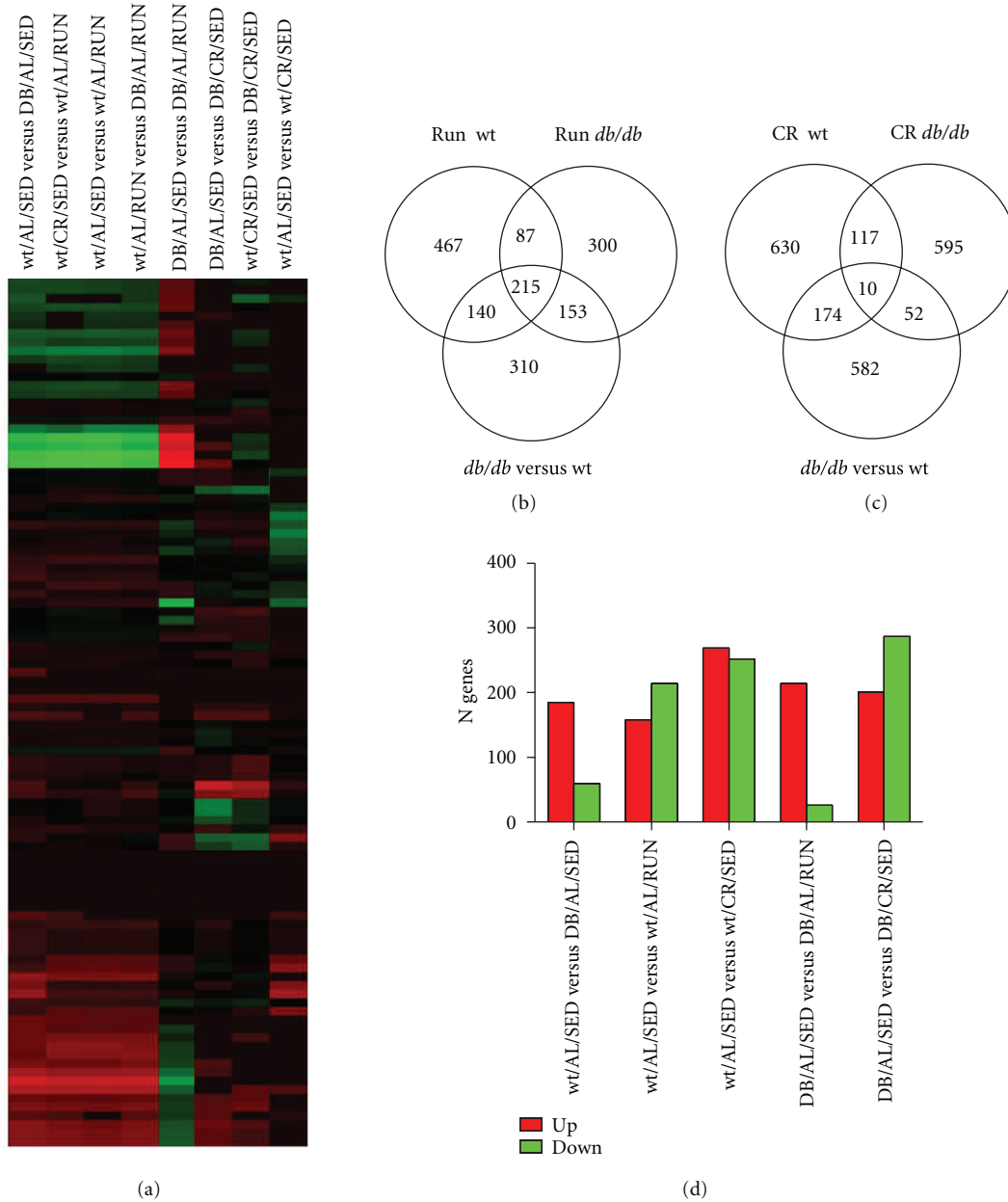


FIGURE 8: *db/db* and C57Bl/6 mice respond differently to exercise and caloric restriction. (a) Heat map profiles showing that *db/db* and C57Bl/6 mice exhibit distinct transcriptional responses to energetic challenges. (b) Venn diagram showing the number of genes expressed following voluntary wheel running in C57Bl/6 and *db/db* mice. (c) Venn diagram showing the number of transcripts responsive to CR in C57Bl/6 and *db/db* mice. (d) Side-by-side comparison of the number of genes that differ between C57Bl/6 and *db/db* mice following running or CR.

CR effects) between WT and *db/db*, only 1.6% of these genes were regulated in the same direction between both mouse backgrounds (Figure 9(a) histogram). In contrast, for the CR paradigm imposition upon the WT and *db/db* mice, a considerably greater percentage of the commonly regulated genes (42.5%) possessed the same polarity of expression across the two mouse genotypes. Taking this coregulated subset of genes [54] we investigated the potential

biological functions of this conserved CR-mediated transcriptomic group (Figure 9(c)). We found that these CR effects upon hypothalamic transcription that were conserved in both WT and *db/db* mice were strongly associated with neuronal development (“nervous system development and function,” “tissue development”) and cellular architecture (“cellular movement,” “cellular assembly and organization”) (Figure 9(c)). Therefore, it appears that the actions of

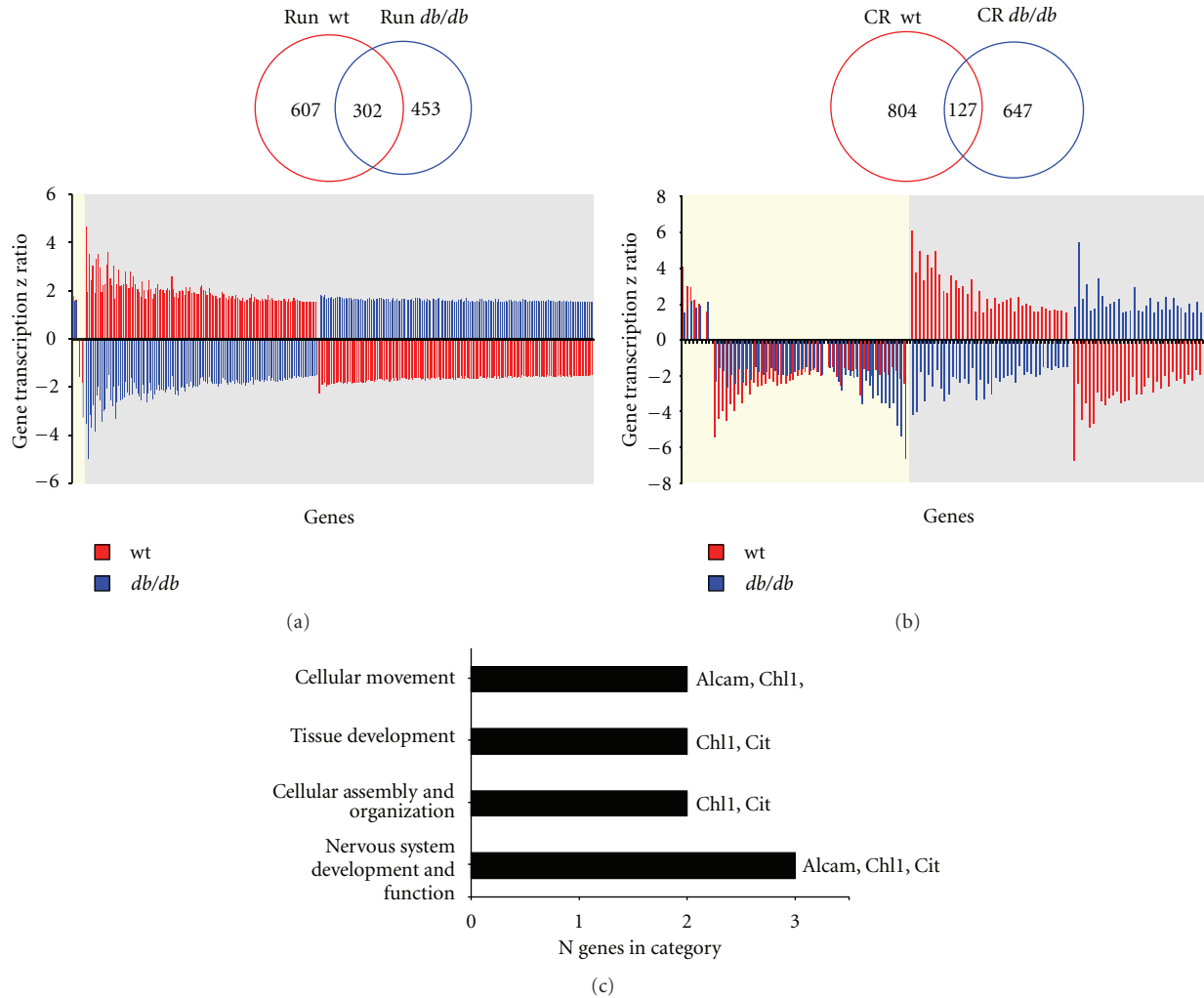


FIGURE 9: Physiological background differentially modulates the effects of exercise and caloric restriction upon hypothalamic transcriptomes. (a) Venn diagram illustrates the number of significantly regulated genes (up- and downregulated) affected by exercise (Run) in WT (red) or *db/db* (blue) animals. The associated histogram indicates the significantly regulated genes common to the effects of exercise in both genetic backgrounds (WT and *db/db*). The yellow area indicates the genes whose direction of regulation was conserved in both genetic backgrounds while the grey area shows the genes that showed reversal of their exercise-mediated expression profile. (b) Venn diagram and associated histogram depict similar data to (a) but for the implementation of CR to the WT (blue) or *db/db* (red) mice. (c) Significantly regulated pathways associated with the conserved CR-induced transcriptional activity in both WT and *db/db* mice.

the antiaging CR paradigm, related to neuronal remodeling/rewiring in the hypothalamus, can be effected in animals of differing metabolic backgrounds. In contrast, the effects of running seem to be transcriptionally sensitive to the animals' metabolic background.

4. Discussion

An absence of leptin receptor signaling in the diabetic *db/db* mice significantly altered the expression of genes associated with excitatory synaptic transmission (*Slc17a6* [58]) and neuronal development (*Pias2* [59]) under *ad libitum*, sedentary conditions. Our data demonstrate that challenges to metabolic homeostasis regulated distinct hypothalamic gene-sets in wild-type and *db/db* mice. Although different

genes were recruited following energetic challenges in wild-type and *db/db* mice, interestingly, transcriptional alterations following running or CR converged on functional pathways associated with nervous system development, suggesting that developmental signaling pathways are important for both neural circuit formation and plasticity in the adult hypothalamus.

Caloric restriction has previously been shown to alter neuronal morphology in the hypothalamus [60] and promotes synaptogenesis in select neuronal populations [23]. In our study, a significant number of genes that play important roles in regulating synaptic plasticity and cytoskeletal motility were differentially expressed in response to energetic challenges (e.g., *Fkbp5* [32]; *Chrd* [40]; *Hcrt* [36]; *App* [61]). While our current study is primarily focused on investigating

global alterations in hypothalamic gene transcription, the fact that we observed numerous significant alterations in the expression of multiple genes involved in regulating plasticity suggests the possibility that challenges to homeostasis can also reorganize circuitry across multiple other brain areas. The hypothalamic effects are likely to develop following an extended period of adaptation to caloric restriction in particular, as few changes in hypothalamic gene expression have been reported in response to shorter durations of CR [62]. As no other studies to date have profiled hypothalamic gene expression in the context of running, it is difficult to speculate on the time course for transcriptional adaptation and further studies are needed to investigate different time courses. Our data suggest that energetic challenges regulate gene transcription across the hypothalamus as a whole, and that the transcriptomic responses are dependent upon the genetic background (i.e., diabetic or nondiabetic). We are planning future studies that will focus on characterizing the genomic responses to energetic challenges that occur in individual hypothalamic nuclei, as this would shed further light upon how distinct neuronal populations respond to energetic challenges. Additionally, we also plan to extend our analyses to investigate overall hypothalamic proteome changes in response to energetic challenges. Due to the highly conserved nature of many neuroendocrine signaling pathways represented in our data, such as insulin/IGF-1, glucocorticoid, and androgen signaling, it is possible that the snapshot of transcriptional alterations captured in these experiments may include mechanisms for the establishment and maintenance of a metabolic set point in other species, with relevance to the prevention and treatment of obesity. Additionally, gaining a greater understanding of how different genetic backgrounds (e.g., diabetic or nondiabetic) can alter hypothalamic transcriptomic responses to energetic challenges, could pave the way for the development of novel therapeutics for the treatment of hypothalamic dysfunction. Our data further suggests that therapeutic caloric restriction paradigms, or pharmacomimetics thereof, may be more readily applicable (compared to exercise paradigms) across different patients with diverse metabolic backgrounds.

Authors' Contributions

A. M. Stranahan and B. Martin contributed equally to this paper.

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Review Article

Sarcopenia and Age-Related Endocrine Function

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Sarcopenia, the age-related loss of skeletal muscle, is characterized by a deterioration of muscle quantity and quality leading to a gradual slowing of movement, a decline in strength and power, and an increased risk of fall-related injuries. Since sarcopenia is largely attributed to various molecular mediators affecting fiber size, mitochondrial homeostasis, and apoptosis, numerous targets exist for drug discovery. In this paper, we summarize the current understanding of the endocrine contribution to sarcopenia and provide an update on hormonal intervention to try to improve endocrine defects. Myostatin inhibition seems to be the most interesting strategy for attenuating sarcopenia other than resistance training with amino acid supplementation. Testosterone supplementation in large amounts and at low frequency improves muscle defects with aging but has several side effects. Although IGF-I is a potent regulator of muscle mass, its therapeutic use has not had a positive effect probably due to local IGF-I resistance. Treatment with ghrelin may ameliorate the muscle atrophy elicited by age-dependent decreases in growth hormone. Ghrelin is an interesting candidate because it is orally active, avoiding the need for injections. A more comprehensive knowledge of vitamin-D-related mechanisms is needed to utilize this nutrient to prevent sarcopenia.

1. Introduction

Age-related declines in muscle mass and strength, known as sarcopenia, are often an important antecedent of the onset of disability in older adulthood. Although the term is applied clinically to denote loss of muscle mass, sarcopenia is often used to describe both a set of cellular processes (denervation, mitochondrial dysfunction, and inflammatory and hormonal changes) and a set of outcomes such as decreased muscle strength, decreased mobility and function [1], increased fatigue, a greater risk of falls [2], and reduced energy needs. Lean muscle mass generally contributes up to ~50% of total body weight in young adults but declines with aging to be 25% at 75–80 years old [3, 4]. The loss of muscle mass is typically offset by gains in fat mass. The loss is most notable in the lower limb muscle groups, with the cross-sectional area of the vastus lateralis being reduced by as much as 40% between the ages of 20 and 80 yr [5].

Several possible mechanisms for age-related muscle atrophy have been described; however, the precise contribution of each is unknown. Age-related muscle loss is a result of

reductions in the size and number of muscle fibers [6] possibly due to a multifactorial process that involves physical activity, nutritional intake, oxidative stress, and hormonal changes [2, 7]. The specific contribution of each of these factors is unknown, but there is emerging evidence that the disruption of several positive regulators (Akt and serum response factor) of muscle hypertrophy with age is an important feature in the progression of sarcopenia [8–10]. In contrast, many investigators have failed to demonstrate an age-related enhancement in levels of common negative regulators (Atrogin-1, myostatin, and calpain) in senescent mammalian muscles.

Several lines of evidence point to inflammation being associated with loss of muscle strength and mass with aging [11]. Animal studies have shown that the administration of interleukin (IL)-6 or tumor necrosis factor (TNF)- α increases skeletal muscle breakdown, decreases the rate of protein synthesis, and reduces plasma concentrations of insulin-like growth factor [12, 13]. In older men and women, higher levels of IL-6 and C-reactive protein (CRP) were associated with a two- to threefold greater risk of losing

more than 40% of grip strength over 3 years [14]. On the other hand, several studies have indicated age-related endocrine defects such as decreases in anabolic hormones (testosterone, estrogen, growth hormone (GH), and insulin-like growth factor-I (IGF-I)) [15–18]. Although hormonal supplementation for the elderly has been conducted on a large scale, it was found not to be effective against sarcopenia and to have minor side effects [9, 10, 15, 16, 19, 20]. In this paper, we summarize the current understanding of the endocrine contribution to sarcopenia and provide an update on practical hormonal intervention for the elderly.

2. The Adaptive Changes in Catabolic Mediators

2.1. *TNF- α* . Inflammation may negatively influence skeletal muscle through direct catabolic effects or through indirect mechanisms (i.e., decreases in GH and IGF-I concentrations, induction of anorexia, etc.) [21]. There is growing evidence that higher levels of inflammatory markers are associated with physical decline in older individuals, possibly through the catabolic effects of these markers on muscle. In an observational study of more than 2000 men and women, *TNF- α* showed a consistent association with declines in muscle mass and strength [22]. The impact of inflammation on the development of sarcopenia is further supported by a recently published animal study showing that a reduction in low-grade inflammation by ibuprofen in old (20 months) rats resulted in a significant decrease in muscle mass loss [23]. An age-related disruption of the intracellular redox balance appears to be a primary causal factor for a chronic state of low-grade inflammation. More recently, Chung et al. [24] hypothesized that abundant nuclear factor- κ B (NF- κ B) protein induced age-related increases in IL-6 and *TNF- α* . Moreover, reactive oxygen species (ROS) also appear to function as second messengers for *TNF- α* in skeletal muscle, activating NF- κ B either directly or indirectly [25]. Indeed, marked production of ROS has been documented in muscle of the elderly [26, 27]. However, it is not clear whether NF- κ B signaling is enhanced with age. Despite some evidence supporting enhanced NF- κ B signaling in type I fibers of aged skeletal muscle, direct evidence for increased activation and DNA binding of NF- κ B is lacking [28, 29]. For example, Phillips and Leeuwenburgh [29] found that neither p65 protein expression nor the binding activity of NF- κ B was significantly altered in the vastus lateralis muscles of 26-month-old rats despite the marked upregulation of *TNF- α* expression in both blood and muscle. Upregulated *TNF- α* expression in serum and muscle seems to enhance apoptosis through increased mitochondrial defects resulting in a loss of muscle fibers [29–31]. It has been shown that *TNF- α* is one of the primary signals inducing apoptosis in muscle.

2.2. *Myostatin*. Myostatin was first discovered during screening for new members of the transforming growth factor- β (TGF- β) superfamily and shown to be a potent negative regulator of muscle growth [32]. Like other family members, myostatin is synthesized as a precursor protein that is cleaved

by furin proteases to generate the active C-terminal dimer. Most, if not all, of the myostatin protein that circulates in blood also appears to exist in an inactive complex with a variety of proteins, including the propeptide [33]. The latent form of myostatin seems to be activated *in vitro* by dissociation from the complex with either acid or heat treatment [33, 34] or by proteolytic cleavage of the propeptide with members of the bone morphogenetic protein-1/tolloid family of metalloproteases [34].

Studies indicate that myostatin inhibits cell cycle progression and reduces levels of myogenic regulatory factors (MRFs), thereby controlling myoblastic proliferation and differentiation during developmental myogenesis [35–37]. Myostatin binds to and signals through a combination of ActRIIA/B receptors on the cell membrane but has higher affinity for ActRIIB. On binding to ActRIIB, myostatin forms a complex with either activin receptor-like kinase (ALK) 4 or ALK5 to activate (phosphorylate) the Smad2/3 transcription factors. Then Smad2/3 are translocated and modulate the nuclear transcription of genes such as MyoD [38] via a TGF- β -like mechanism. More recently, the IGF-I-Akt-mTOR (mammalian target of rapamycin) pathway, which mediates both differentiation in myoblasts and hypertrophy in myotubes, has been shown to inhibit myostatin-dependent signaling. Blockade of the Akt-mTOR pathway, using siRNA to RAPTOR, a component of TORC1 (TOR signaling complex 1), facilitates myostatin's inhibition of muscle differentiation because of an increase in Smad2 phosphorylation [39]. In contrast, Smad2/3 inhibition promotes muscle hypertrophy partially dependent on mTOR signaling [40].

Several researchers have investigated the effect of inhibiting myostatin to counteract sarcopenia using animals [41, 42]. Lebrasseur et al. [41] found that treatment with a mouse chimera of antihuman myostatin antibody (24 mg/Kg, 4 weeks), a drug for inhibiting myostatin, elicited a significant increase in muscle mass and in running performance probably due to decreased levels of phosphorylated Smad3 and Muscle ring finger-1 (MuRF-1). More recently, Murphy et al. [42] showed, by way of once weekly injections, that a lower dose of this anti-human myostatin antibody (10 mg/Kg) significantly increased the fiber cross-sectional area (by 12%) and *in situ* muscle force (by 35%) of aged mice (21 months old). These findings highlight the therapeutic potential of antibody-directed myostatin inhibition for sarcopenia by inhibiting protein degradation. Although many researchers expect myostatin levels to be increased not only in muscle but also in serum, blood myostatin levels have not been shown to increase with age [43].

2.3. *Glucocorticoid*. Glucocorticoid-associated atrophy appears to be specific to type II or phasic muscle fibers. In a study of controlled hypercortisolaemia in healthy men [44], experimental inactivity increased the catabolic effect of glucocorticoids, suggesting that an absence of mechanical signals potentiates the effect. The mechanism of glucocorticoid-induced atrophy may involve upregulated expression of myostatin and glutamine synthetase, the latter

via the glucocorticoid receptor's interaction with the glutamine synthetase promoter [45]. Glucocorticoids inhibit the physiological secretion of GH and appear to induce IGF-I activity in target organs. Changes in steroid-induced glutamine synthetase represent a potential mechanism of action, and dose-dependent inhibition of glutamine synthetase by IGF-I was observed in rat L6 cells [46].

The increased incidence of various diseased states during aging is associated with the hypersecretion of glucocorticoids [47, 48]. In addition, when adult (7-month-old) and aged (22-month-old) rats received dexamethasone (approximately 500 µg/Kg body weight/day) in their drinking water for 5-6 days, muscle wasting was much more rapid in aged animals [47]. Furthermore, glucocorticoids induced prolonged leucine resistance to muscle protein synthesis in old rats [49]. Still, it remains to be directly elucidated, using pharmacological inhibitors for glucocorticoids, whether age-related increases in serum glucocorticoid levels actually inhibit protein synthesis and/or enhance protein degradation.

2.4. Interleukin-6 and CRP. IL-6 and CRP, known as "geriatric cytokines," are multifunctional cytokine produced in situations of trauma, stress, and infection. During the aging process, levels of both IL-6 and CRP in plasma become elevated. The natural production of cytokines is likely beneficial during inflammation, but overproduction and the maintaining of an inflammatory state for long periods of time, as seen in elderly individuals, are detrimental [50, 51]. A number of authors have demonstrated that a rise in plasma levels of proinflammatory cytokines, especially IL-6, and proteins under acute conditions is associated with a reduction in mobility as well as a reduced capacity to perform daily activities, the development of fragility syndrome, and increased mortality rates [50–52]. In older men and women, higher levels of IL-6 and CRP were associated with a two- to threefold greater risk of losing more than 40% of grip strength over 3 years [14]. In contrast, there were no longitudinal associations between inflammatory markers and changes in grip strength among high functioning elderly participants from the MacArthur Study of Successful Ageing [53]. More recently, Hamer and Molloy [54] demonstrated, in a large representative community-based cohort of older adults (1,926 men and 2,260 women (aged 65.3 ± 9.0 years)), that CRP was associated with poorer hand grip strength and chair stand performance in women but only chair stand performance in men. In addition, Haddad et al. [55] demonstrated atrophy in the tibialis anterior muscle of mice following the injection of relatively low doses of IL-6.

In a recent randomized trial that employed aerobic and strength training in a group of elderly participants, significant reductions in various inflammatory markers (IL-6, CRP, and IL-18) were observed for aerobic but not strength training [56]. In contrast, combined resistance and aerobic training that increased strength by 38% resulted in significant reductions in CRP [57]. More descriptive data appears to be needed whether IL-6 and CRP have an actual catabolic effect in sarcopenic muscle.

3. Anabolic Hormones in Sarcopenic Muscle

3.1. Testosterone. In males, levels of testosterone decrease by 1% per year, and those of bioavailable testosterone by 2% per year from age 30 [16, 58, 59]. In women, testosterone levels drop rapidly from 20 to 45 years of age [60]. Testosterone increases muscle protein synthesis [61], and its effects on muscle are modulated by several factors including genetic background, nutrition, and exercise [62].

Numerous studies of treatment with testosterone in the elderly have been performed over the past few years [63–66]. In 1999, Snyder et al. [66] suggested that increasing the level of testosterone in old men to that seen in young men increased muscle mass but did not result in functional gains in strength. Systemic reviews of the literature [67] have concluded that testosterone supplementation attenuates several sarcopenic symptoms including decreases in muscle mass [64–66] and grip strength [63]. For instance, a recent study of 6 months of supraphysiological dosage of testosterone in a randomized placebo-controlled trial reported increased leg lean body mass and leg and arm strength [68]. Although there are significant increases in strength among elderly males given high doses of testosterone, the potential risks may outweigh the benefits. Risks associated with testosterone therapy in older men include sleep apnea, thrombotic complications, and the increased risk of prostate cancer [69].

These side effects have driven the necessity for drugs that demonstrate improved therapeutic profiles. Novel, nonsteroidal compounds, called selective androgen receptor modulators, have shown tissue-selective activity and improved pharmacokinetic properties. Whether these drugs are effective in treating sarcopenia has yet to be shown [70]. Dehydroepiandrosterone (DHEA) is marketed as a nutritional supplement in the USA and is available over the counter. Unlike testosterone and estrogen, DHEA is a hormone precursor which is converted into sex hormones in specific target tissues [71]. However, supplementation of DHEA in aged men and women resulted in an increase in bone density and testosterone and estradiol levels, but no changes in muscle size, strength, or function [72, 73].

3.2. Estrogen. It has been hypothesized that menopause transition and the subsequent decline in estrogen may play a role in muscle mass loss [7, 18]. Van Geel et al. [74] reported a positive relationship between lean body mass and estrogen levels. Similarly, Iannuzzi-Sucich et al. [75] observed that muscle mass is correlated significantly with plasma estrone and estradiol levels in women. However, Baumgartner et al. [76] reported that estrogen levels were not associated with muscle mass in women aged 65 years and older. The mechanisms by which decrease in estrogen levels may have a negative effect on muscle mass are not well understood but may be associated with an increase in proinflammatory cytokines, such as TNF- α and IL-6, which might be implicated in the apparition of sarcopenia [77]. Furthermore, estrogen could have a direct effect on muscle mass since it has been shown that skeletal muscle has estrogen beta-receptors on the cell membrane [78].

Therefore, a direct potential mechanistic link could exist between low estrogen levels and a decrease in protein synthesis. Further studies are needed to investigate this hypothesis. Nevertheless, before reaching a conclusion on the contribution of estrogens to the onset of sarcopenia, it would be important to measure urinary estrogen metabolites since a relationship between breast cancer and urinary estrogen metabolites has been shown [79].

3.3. GH. Growth hormone (GH) is a single-chain peptide of 191 amino acids produced and secreted mainly by the somatotrophs of the anterior pituitary gland. GH coordinates the postnatal growth of multiple target tissues, including skeletal muscle [80]. GH secretion occurs in a pulsatile manner with a major surge at the onset of slow-wave sleep and less conspicuous secretory episodes a few hours after meals [81] and is controlled by the actions of two hypothalamic factors, GH-releasing hormone (GHRH), which stimulates GH secretion, and somatostatin, which inhibits GH secretion [82]. The secretion of GH is maximal at puberty accompanied by very high circulating IGF-I levels [83], with a gradual decline during adulthood. Indeed, circulating GH levels decline progressively after 30 years of age at a rate of ~1% per year [84]. In aged men, daily GH secretion is 5- to 20-fold lower than that in young adults [85]. The age-dependent decline in GH secretion is secondary to a decrease in GHRH and to an increase in somatostatin secretion [86].

With respect to the somatomedin hypothesis, the growth-promoting actions of GH are mediated by circulating or locally produced IGF-I [87]. GH-induced muscle growth may be mediated in an endocrine manner by circulating IGF-I derived from liver and/or in an autocrine/paracrine manner by direct expression of IGF-I from target muscle via GH receptors on muscle membranes. The effects of GH administration on muscle mass, strength and physical performance are still under debate [19]. In animal models, GH treatment is very effective at inhibiting sarcopenic symptoms such as muscle atrophy and decreases in protein synthesis particularly in combination with exercise training [88]. The effect of GH treatment for elderly subjects is controversial. Some groups demonstrated an improvement in strength after long-term administration (3–11 months) of GH [89]. In contrast, many researchers have found that muscle strength or muscle mass did not improve on supplementation with GH [19, 89]. One recent study reported a positive effect for counteracting sarcopenia after the administration of both GH and testosterone [90]. Several reasons may underlie the ineffectiveness of GH treatment in improving muscle mass and strength in the elderly, such as a failure of exogenous GH to mimic the pulsatile pattern of natural GH secretion or the induction of GH-related insulin resistance. In addition, reduced mRNA levels of the GH receptor in skeletal muscle have been observed in older versus younger healthy men, exhibiting a significant negative relationship with myostatin levels [91]. It should also be considered that the majority of the trials conducted on GH supplementation have reported a high incidence of side

effects, including soft tissue edema, carpal tunnel syndrome, arthralgias, and gynecomastia, which pose serious concerns especially in older adults. Therefore, one should pay very careful attention when administering GH to the elderly.

There is evidence that the age-associated decline in GH levels in combination with lower IGF-I levels contributes to the development of sarcopenia [92]. IGF-I is perhaps the most important mediator of muscle growth and repair [93] possibly by utilizing Akt-mTOR-p70S6K (p70 ribosomal protein S6 kinase) signaling. Although the transgenic approach of upregulating IGF-I expression in skeletal muscle would be appropriate for inhibiting sarcopenia, the administration of IGF-I to the elderly has resulted in controversial findings on muscle strength and function [94]. The ineffectiveness may be attributable to age-related insulin resistance to amino acid transport and protein synthesis [95] or a marked decrease in IGF-I receptors [96, 97] and receptor affinity for IGF-I [98] in muscle with age. Wilkes et al. [99] demonstrated a reduced effect of insulin on protein breakdown in the legs in older versus younger subjects probably due to the blunted activation of Akt by insulin. More comprehensive reviews on insulin resistance in sarcopenia can be found elsewhere [95].

3.4. Ghrelin. Ghrelin is a 28-amino-acid peptide mainly produced by cells in the stomach, intestines, and hypothalamus [100]. Ghrelin is a natural ligand for the GH-secretagogue receptor (GHS-R), which possesses a unique fatty acid modification, an n-octanoylation, at Ser 3 [101]. Ghrelin plays a critical role in a variety of physiological processes, including the stimulation of GH secretion and regulation of energy homeostasis by stimulating food intake and promoting adiposity via a GH-independent mechanism [100]. In contrast, ghrelin inhibits the production of anorectic proinflammatory cytokines, including IL-1 β , IL-6, and TNF- α [102]. Because of their combined anabolic effects on skeletal muscle and appetite, ghrelin and low-molecular-weight agonists of the ghrelin receptor are considered attractive candidates for the treatment of cachexia [103]. For example, Nagaya et al. [104] gave human ghrelin (2 μ g/Kg twice daily intravenously) for 3 weeks to cachexic patients with chronic obstructive pulmonary disease in an open-label study. After ghrelin therapy, significant increases from baseline measurements were observed for body weight, lean body mass, food intake, hand grip strength, maximal inspiratory pressure, and Karnofsky performance score [104]. In another unblinded study, the same group demonstrated that treatment with human ghrelin (2 μ g/Kg twice daily intravenously, 3 weeks) significantly improved several parameters (eg., lean body mass measured by dual-energy X-ray absorption and left ventricular ejection fraction) in 10 patients with chronic heart failure [105]. In a 1-year placebo-controlled study in healthy older adults over the age of 60 years given an oral ghrelin-mimetic (MK-677), an increase in appetite was observed [106]. The study did not show a significant increase in strength or function in the ghrelin-mimetic treatment group, when compared to the placebo group; however, a tendency was observed [106]. As pointed out in a recent

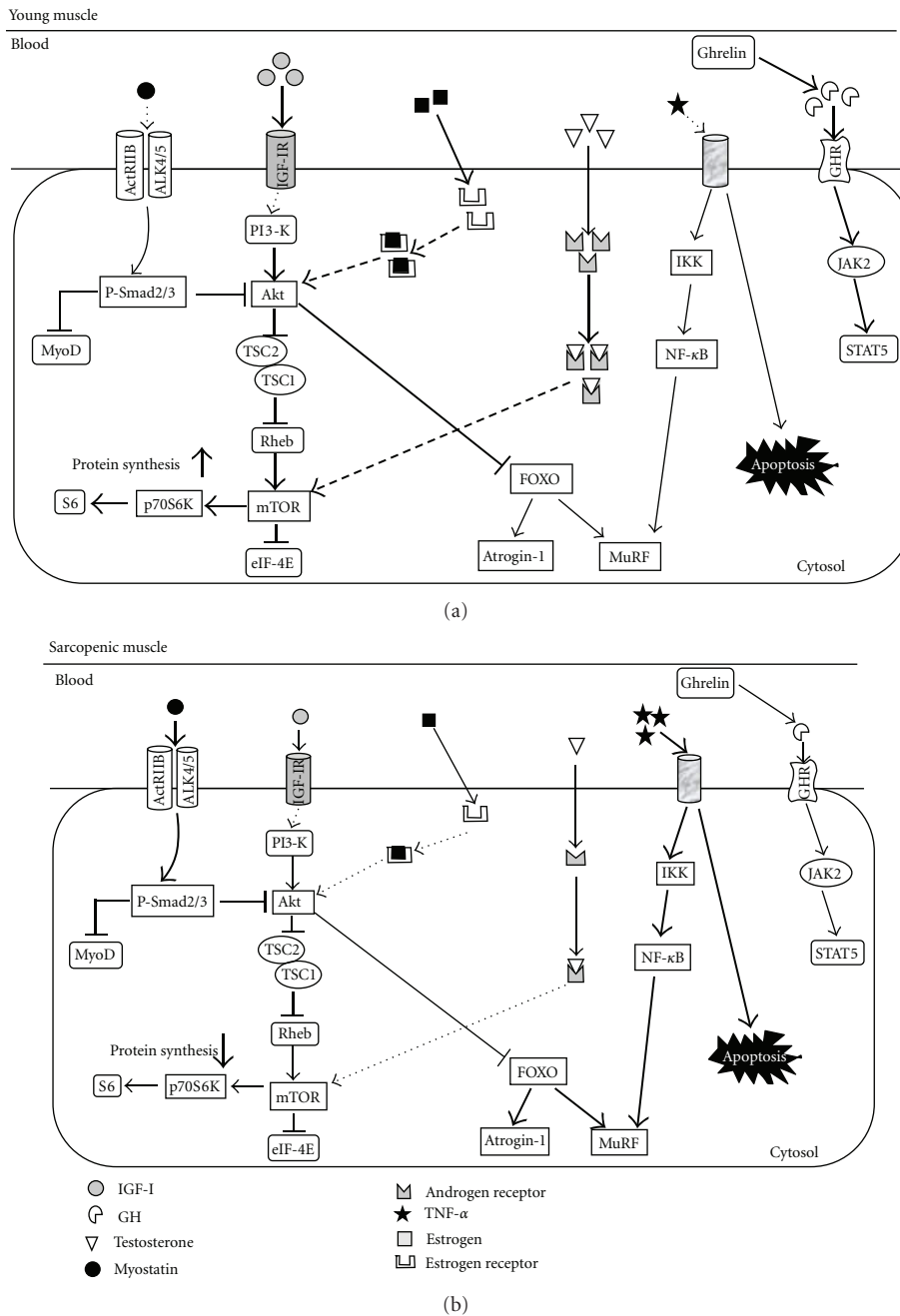


FIGURE 1: (a) In young muscle, abundant serum IGF-I can stimulate protein synthesis by activating Akt/mTOR/p70S6K pathway. Akt blocks the nuclear translocation of FOXO to inhibit the expression of Atrogin-1 and MuRF and the consequent protein degradation. Abundant serum GH, which is induced by ghrelin, activates JAK2-STAT5 signaling to promote muscle-specific gene transcription necessary to hypertrophy. In young muscle, testosterone and estrogen bind these intramuscular receptors (androgen receptor and estrogen receptor (α and β)), and activate mTOR and Akt, respectively. Lower serum amount of myostatin and TNF- α failed to activate signaling candidates (Smad 2/3, NF- κ B, etc.) enhancing protein degradation. (b) In sarcopenic muscle, myostatin signals through the activin receptor IIB (ActRIIB), ALK4/5 heterodimer seems to activate Smad2/3 and blocking of MyoD transactivation in an autoregulatory feedback loop. Abundant activated Smad2/3 inhibit protein synthesis probably due to blocking the functional role of Akt. The increased blood TNF- α elevates the protein degradation through IKK/NF- κ B signaling and enhance an apoptosis. Lower serum amount of IGF-I, GH, and anabolic hormones (testosterone and estrogen) failed to activate signaling candidates (Akt, mTOR, STAT5, etc.) enhancing protein synthesis. The impaired regulation of FOXO by Akt results in abundant expression of Atrogin-1 and MuRF and the consequent protein degradation in sarcopenic muscle.

review by Nass et al. [20], the use of this compound induces the potential deterioration of insulin sensitivity and development of diabetes mellitus in older adults with impaired glucose tolerance. Figure 1 provides an overview of several regulators for muscle mass in both young and sarcopenic mammalian muscles.

3.5. Vitamin D. Vitamin D has been traditionally considered a key regulator of bone metabolism and calcium and phosphorus homeostasis through negative feedback with the parathyroid hormone [107, 108]. It is also well established that vitamin D deficiency causes rickets in children and osteomalacia and osteoporosis in adults. A large and growing body of evidence suggests that vitamin D is not only necessary for bone tissue and calcium metabolism but may also represent a crucial determinant for the development of major (sub)clinical conditions and health-related events [107, 109].

Today, approximately 1 billion, mostly elderly people, worldwide have vitamin D deficiency. The prevalence of low vitamin D concentrations in subjects older than 65 years of age has been estimated at approximately 50% [110–112], but this figure is highly variable because it is influenced by sociodemographic, clinical, therapeutic, and environmental factors. Similarly there is an age-dependent reduction in vitamin D receptor expression in skeletal muscle [113]. Prolonged vitamin D deficiency has been associated with severe muscle weakness, which improves with vitamin D supplementation [114]. The histological examination of muscle tissue from subjects with osteomalacia is characterized by increased interfibrillar space, intramuscular adipose tissue infiltrates, and fibrosis [115]. Interestingly, muscle biopsies performed before and after vitamin D supplementation have documented an increased number and sectional area of type II (or fast) muscle fibers [113, 116].

A large body of evidence currently demonstrates that low vitamin D concentrations represent an independent risk factor for falls in the elderly [117–119]. Supplementation with vitamin D in double-blind randomized-controlled trials has been shown to increase muscle strength and performance and reduce the risk of falling in community-living elderly and nursing home residents with low vitamin D levels [120–124]. In contrast, several groups found no positive effect of vitamin D supplementation on fall event outcomes [125–127]. Cesari et al. [128] attributed these contradictory findings to the selection criteria adopted to recruit study populations, adherence to the intervention, or the extreme heterogeneity of cut-points defining the status of deficiency. A more comprehensive knowledge on vitamin-D-related mechanisms may provide a very useful tool preventing muscle atrophy for older persons (sarcopenia).

4. Conclusion

Given the current and future demographic age shift in the world's population, intense research in this area is imperative. Decreases in muscle mass have been shown to be a key element in the development of frailty. Currently, resistance

training combined with amino-acid-containing supplements would be the best way to prevent age-related muscle wasting and weakness. Comprehensive trials have demonstrated that supplementation with GH, IGF-I, or estrogen has a minor sarcopenia-inhibiting effect. Testosterone supplementation in large amounts improves muscle defects with aging but has several side effects. Ghrelin-mimetics which have the ability to increase caloric intake as well as to increase lean body mass in the older population could be potentially beneficial and reverse the catabolic state associated with sarcopenia. Myostatin inhibition seems to be an intriguing strategy for attenuating sarcopenia as well as muscular dystrophy.

Abbreviations

ActRIIB:	Activin type IIB receptor
ALK:	Activin receptor-like kinase
CRP:	C-reactive protein
DHEA:	Dehydroepiandrosterone
GH:	Growth hormone
GHRH:	Growth hormone releasing hormone
IGF-I:	Insulin-like growth factor-I
IL:	Interleukin
mTOR:	Mammalian target of rapamycin
MuRF-1:	Muscle ring finger-1
NF- κ B:	Nuclear factor-kappaB
p70S6K:	p70 ribosomal protein S6 kinase
ROS:	Reactive oxygen species
TNF- α :	Tumor necrosis factor- α
TORC1:	TOR signaling complex 1.

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Review Article

Pancreatic Function, Type 2 Diabetes, and Metabolism in Aging

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Aging is a risk factor for impaired glucose tolerance and diabetes. Of the reported 25.8 million Americans estimated to have diabetes, 26.9% are over the age of 65. In certain ethnic groups, the proportion is even higher; almost 1 in 3 older Hispanics and African Americans and 3 out of 4 Pima Indian elders have diabetes. As per the NHANES III (Third National Health and Nutrition Examination) survey, the percentage of physician-diagnosed diabetes increased from 3.9% in middle-aged adults (40–49 years) to 13.2% in elderly adults (≥ 75 years). The higher incidence of diabetes is especially alarming considering that diabetes in itself increases the risk for multiple other age-related diseases such as cancer, stroke, cardiovascular diseases, Parkinson's disease, and Alzheimer's disease (AD). In this review, we summarize the current evidence on how aging affects pancreatic β cell function, β cell mass, insulin secretion and insulin sensitivity. We also review the effects of aging on the relationship between insulin sensitivity and insulin secretion. Understanding the mechanisms that lead to impaired glucose homeostasis and T2D in the elderly will lead to development of novel treatments that will prevent or delay diabetes, substantially improve quality of life and ultimately increase overall life span.

1. Introduction

Aging is an important risk factor for metabolic disorders, including obesity, impaired glucose tolerance, and type 2 diabetes (T2D). Diabetes and its complications remain major causes of morbidity and mortality in the USA [1]. It has been reported that the prevalence of T2D increases with age and peaks at 60–74 [2–4]. Almost one third of the elderly have diabetes and three quarters have diabetes or prediabetes [5]. The higher incidence of diabetes is especially alarming considering that diabetes in itself increases the risk for multiple other age-related diseases such as cardiovascular disease (CVD), atherosclerosis, stroke, Alzheimer disease (AD), Parkinson's disease, nonalcoholic fatty liver disease (NAFLD), and cancer [6]. The pathogenesis of T2D in aging is characterized by two major features: peripheral insulin resistance and impaired insulin secretion from β cells [7]. Here, we review how aging predisposes to diabetes and

impaired glucose tolerance through effects on insulin secretion and insulin action.

2. Aging and Insulin Secretion

Age-related defects in insulin secretion have been demonstrated in rodents as well as humans. Glucose and amino acid are major stimuli for insulin release from the pancreatic β cell. With aging, there is a decrease in insulin secretion following stimulation with glucose as well as amino acid arginine [8]. Decrease in glucose-stimulated insulin secretion (GSIS) *in vivo* has been shown in rodents using the state-of-the-art hyperglycemic clamps [9]. In humans, disorderly insulin release, a decrease in insulin pulse amplitudes, and decreased response to glucose oscillations as well as alterations in insulin clearance have all been demonstrated [10]. When the insulin secretory defects are superimposed over an increased need for insulin as in old age, impaired glucose

homeostasis, glucose intolerance, and diabetes can result. We have demonstrated in rodent models that older rodents are unable to increase insulin secretion in proportion to the increased demands imposed by insulin resistance [9], thus contributing to impaired glucose tolerance. Similarly, studies in humans have demonstrated a secretory defect that is consistently observed even after controlling for insulin action. Many factors contribute to the decrease in insulin secretion in aging, including the age-associated loss of Sirt1-mediated GSIS [11], decreased β -cell sensitivity to circulating incretins [10], age-associated decrease in mitochondrial function, as well as increased oxidative stress [12]. In this section of the review, we will specifically address age-related decline in various aspects of β -cell function and mass that could contribute to the observed defects in insulin secretion.

2.1. Aging and Glucose Stimulated Insulin Secretion. Insulin, secreted from pancreatic β cells, is the major hormone in regulating glucose homeostasis. The secretion of insulin from β cells is a complex process involving the integration of multiple stimuli, such as nutrients, hormones, neurotransmitters, and drugs, but the primary stimulus for insulin secretion is circulating glucose. Aging is associated with a marked decline in GSIS in both humans and rodents [13, 14] and the impairment of GSIS is one of the hallmarks of T2D [15]. It is widely accepted that there are five important and “regulatable” steps involved in glucose-induced insulin secretion (as shown in Figure 1): (1) glucose is transported into the β cells through the translocation of the glucose transporters (GLUTs), especially GLUT2; (2) generation of ATP through the oxidation of glucose; (3) elevation the ratio of ATP/ADP induces closure of cell-surface ATP-sensitive K^+ (K_{ATP}) channels, leading to cell membrane depolarization; (4) extracellular Ca^{2+} influx into the β cell; (5) a rise in cytosolic Ca^{2+} triggering the exocytosis of insulin granules. We will systematically address how aging affects each of these processes.

2.1.1. Aging and Glucose Transporters in β Cells. As shown in Figure 1, initiation of the glucose transport is the first step that links glucose metabolism to insulin release in the β cell. GLUT2 is the major glucose transporter expressed in pancreatic β cells and ensures large bidirectional fluxes of glucose and other dietary sugars, such as fructose and galactose, in and out the cell due to its low affinity and high capacity. Glucose transport is an earlier event in GSIS. Loss of pancreatic β -cell GLUT2 expression in humans is associated with hyperglycemia and impaired GSIS [16], and the loss of GSIS directly correlates with decreased expression of the β cell GLUT2 in several rodent T2D models [16]. Hou et al. observed that high extracellular glucose concentrations enhance GLUT2 endocytosis, which leads to the insulin secretion in GLUT2 overexpressed β -cell line [17]. However, the internalized GLUT2 protein undergoes rapid degradation induced by chronic high-glucose stimulation, which indicates that hyperglycemia directly affects β cells function [17]. Moreover, mice lacking GLUT2 in pancreatic β cell display an almost complete absence of first phase glucose stimulated

insulin secretion [16]. Taken together, these studies suggest that GLUT2 is essential for GSIS and lack of GLUT2 causes hyperglycemia. GLUT2 expression was diminished in very old animals compared with juvenile and adult rhesus monkeys [18], implying the potential connection between age and GLUT2 expression level. Age-associated decrease in expression of GLUT2 has been demonstrated in aged rodent models along with other β cell specific genes [19]. The study by Ohneda et al. shows that GLUT2 is underexpressed with increased age; however, neither the magnitude of the underexpression of GLUT2 nor of the reduction in GLUT2 transport function in islets of Goto-Kakizaki (GK) rats is sufficient by itself to explain the profound reduction in GSIS [20]. Compared to rodents, recent evidence demonstrates that human β cells express three glucose transporters, GLUT1, 2, and 3 [21]. The higher levels of GLUT1 and GLUT3 may introduce differences in the regulation of glucose sensing in humans versus rodent islets.

2.1.2. Effects of Age on β -Cell Glucose Oxidation. After uptake into β cells, glucose undergoes oxidation and eventually generates ATP in cytosol and mainly mitochondria via the citric acid cycle also known as the tricarboxylic acid (TCA) cycle, or the Krebs cycle (Figure 1). In the pancreatic β cells, glucose oxidation results in the increase of ATP production which is required for insulin secretion. Islets from T2D patients exhibit lower ATP content and blunted GSIS, implicating the mitochondria in the pathogenesis of β cell dysfunction [22]. The metabolism of glucose is initiated by its phosphorylation by glucokinase (GCK), a member of a family of evolutionary and structurally related hexokinases. The reaction catalyzed by GCK is the first reaction in glycolysis as well as the first rate-limiting reaction in the metabolism of glucose [23]. GCK mRNA level is markedly decreased in diabetic compared to the normal rats. The gene expression level of GCK was significantly increased with age in healthy rats [24], suggesting a potential mechanism by which β cells attempt to overcome age-associated glucose intolerance and insulin resistance. Interestingly, there is evidence to show that glucose oxidation rates are lower in older animals [25]. By using [1- ^{14}C] and [6- ^{14}C] glucose-incubated islets isolated from pancreases of 2-month and 12-month-old rats, G. M. Reaven and P. D. Reaven demonstrated that the amount of glucose converted to CO_2 by islets from 12-month-old rats was only half that of 2-month-old rats [25]. In humans, there is a lower glucose oxidation but higher lipid oxidation rates in elderly than in the young, suggesting that an enhanced Randle cycle may play a major role in producing a reduction in insulin-mediated glucose oxidation [26].

MacDonald has pointed out that pancreatic islets contain 40–70 times the activity of mitochondrial glycerophosphate dehydrogenase (GPDH) compared to other tissues [27], implicating the potential importance of GPDH in islets. Since GPDH plays a crucial role in transporting and reducing equivalents from cytosol to mitochondria, a decrease in its activity leads to an accumulation of cytosolic NADH and consequently, an increase in the cytosolic NAD/NAD $^+$ ratio, a decrease in glycolysis, and a reduction in GSIS. An ap-

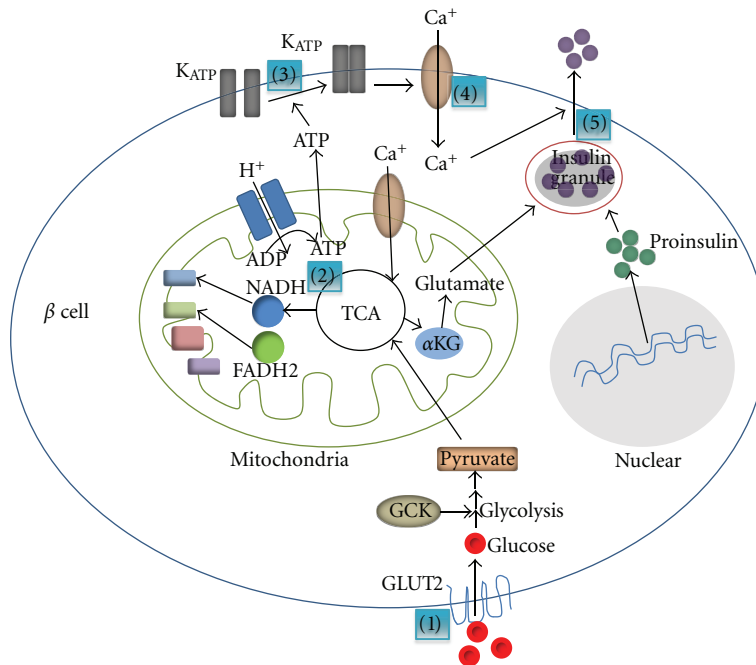


FIGURE 1: The processes of GSIS. (1) Glucose is transported into the β cells through the translocation of the glucose transporters, especially GLUT2; (2) generation of ATP through the oxidation of glucose; (3) elevation the ratio of ATP/ADP induces closure of cell-surface ATP-sensitive K^+ (K_{ATP}) channels, leading to cell membrane depolarization; (4) extracellular Ca^{2+} influx into the β cell; (5) a rise in cytosolic Ca^{2+} triggers the exocytosis of insulin granules. K_{ATP} : ATP-sensitive K^+ channels, GLUT2: glucose transporter 2, GSK: phosphorylation by glucokinase, G6PDH: glycerophosphate dehydrogenase, TCA cycle: tricarboxylic acid cycle, NADH: reduced form of Nicotinamide adenine dinucleotide, FADH₂: reduced forms of flavin adenine dinucleotide, α KG: alpha-ketoglutarate, ADP: adenosine diphosphate, ATP: Adenosine-5'-triphosphate.

proximate 50% reduction in the activity of G6PDH in islets of 12-month-old compared with 2-month-old rats [28], suggesting a role for G6PDH in diminished GSIS in aging.

2.1.3. Aging and Calcium and Potassium Channels. Increased ATP/ADP ratio from glucose oxidation reduces the whole cell K^+ permeability, leading to cell membrane depolarization and extracellular Ca^{2+} influx into the β cell. These changes are thought to be mediated through the ATP-sensitive K^+ (K_{ATP}) channels and voltage-dependent Ca^{2+} channels (Figure 1). Elevation of cytosolic free Ca^{2+} concentration provides a link with the insulin exocytotic process. Studies from Ammon and colleagues have shown that raising the glucose concentration from 3 to 5.6 and 16.7 mM had no effect on K^+ efflux from islets of 24-month-old male rats whereas that from 24-month-old female rats were decreased. Also, net uptake of Ca^{2+} was significantly diminished in islets of 24-month-old compared to islets of 3-month-old rats. In the presence of 16.7 mM of glucose, islets of 24-month-old rats exhibited only 60–70% of the insulin release obtained with islets from 3-month-old rats. These data suggest that the decreased insulin secretory response to glucose during old age is due, at least in part, to inadequate inhibition of K^+ efflux and diminished net uptake of Ca^{2+} [29]. However, it is important to consider that the differences in ion fluxes could relate to age-related deficits in the steps leading up to ion flux such as decreased glucose transport and oxidation.

2.1.4. Effects of Age on Insulin Granule Exocytosis. The final step of insulin secretion is the exocytosis of insulin granules (Figure 1). Insulin is stored in large dense core vesicles (LDCVs), also called insulin granule, and released by exocytosis, a multistage process involving vesicle trafficking, docking, and eventually fusion with the plasma membrane [30]. Calcium constitutes the major stimulus for exocytosis. Ca^{2+} regulates several steps in exocytosis, such as the size of vesicle pools, the fusion event, and the size of the fusion pore, and may act on distinct protein targets [31, 32]. Since the net uptake of Ca^{2+} is decreased with age [29], it is reasonable to speculate that insulin granule exocytosis is also inhibited by age. To date, insulin secretion is known to involve the same soluble N-ethylmaleimide, sensitive factor attachment protein receptor (SNARE) isoforms as those utilized in synaptic vesicle exocytosis and neurotransmitter release, namely, Syntaxin 1, vesicle-associated membrane protein (VAMP) 2, and synaptosomal-associated protein of 25 kDa (SNAP-25) [33–37]. The exocytosis is induced by the pairing of SNARE proteins on the vesicle membrane, termed v-SNARE (such as VAMP2), with cognate proteins on the target membrane, the t-SNAREs (syntaxin and SNAP-25). In addition, other proteins such as munc18/sec1 and munc13 have also been reported to be involved in the regulation of insulin granule exocytosis [38, 39]. Vanguilder and colleagues showed that neurotransmission-regulating proteins such as VAMP2, Syntaxin1, and SNAP-25 decline with age in

hippocampal synaptosomes in rats. Altered synaptic protein expression may decrease stimulus-induced neurotransmission and vesicle replenishment during prolonged or intense stimulation, two processes that are necessary for learning and the formation and perseverance of memory [40]. However, it is still unclear whether these proteins also decline with age in pancreatic β cells. Further studies are needed to address this question.

2.2. Aging and β -Cell Mass. The proliferation and apoptosis of β cells and islet neogenesis are three major factors that tightly regulate β -cell mass. As shown in Figure 2, in this section, we will review the effects of aging on these factors.

2.2.1. Apoptosis and Proliferation. Pancreatic β -cell mass is mainly controlled by the balance of cell proliferation and apoptosis. It has been shown that age correlates with decreased proliferative activity and enhanced sensitivity to glucose-induced β -cell apoptosis [41] (Figure 2). In cultured islets from 2 to 3-month-old rats, increasing glucose from 5.5 to 11.1 mM decreased β -cell apoptosis and increased β -cell proliferation, which was further augmented when glucose was increased to 33.3 mM. In contrast, in islets from 7 to 8-month-old rats, increasing glucose concentrations from 5.5 to 33.3 mM induced a linear increase in β -cell death and a decrease in proliferation. This has also been observed in cultivated human islets where age correlated positively with the sensitivity to glucose-induced β cell apoptosis and negatively to baseline proliferation [41]. It is reported that there is a three-to-ten-fold increase in β -cell apoptosis in diabetic patients compared to nondiabetic individuals as detected by TUNEL staining [42]. Maedler et al. demonstrated that high glucose induces apoptosis in human pancreatic β cells through inducing the expression of Fas and activation of caspase 8 and 3 [43]. Reers et al. report that relative β -cell volume in human pancreatic islets remains constant with aging and β -cell replication decreases age dependently, while β -cell apoptosis does not change significantly [44]. However, the most recent study performed on 4-month, 14-month and, 24-month-old Wistar rats has shown that there is a significant reduction in β -cell proliferation and increase in apoptosis in β cells in the islets [45]. The study also showed that age associated decreases in activities of many antioxidant enzymes and suggests that an increase in oxidative stress in the β cells contributes to the increased apoptosis [45].

Islet amyloid polypeptide (IAPP) or amylin is cosecreted with insulin from β cells [46]. Amylin suppresses glucagon secretion and helps to regulate glucose homeostasis. In insulin-resistant states, hypersecretion of insulin results in increased cosecretion of amylin. Careful analyses show that the amylin could aggregate into amyloid plaques that increase β -cell apoptosis leading to reduced islet volume and β -cell mass [47], and subsequent diabetes [48]. Accumulating evidence implicates toxic IAPP oligomers in the mediation of β cell apoptosis in T2D. In support, freshly dissolved human IAPP (hIAPP, but not rodent IAPP) induces apoptosis when added to cells in culture [49]. β -cell-specific transgenic overexpression of hIAPP result in hyperglycemia in the

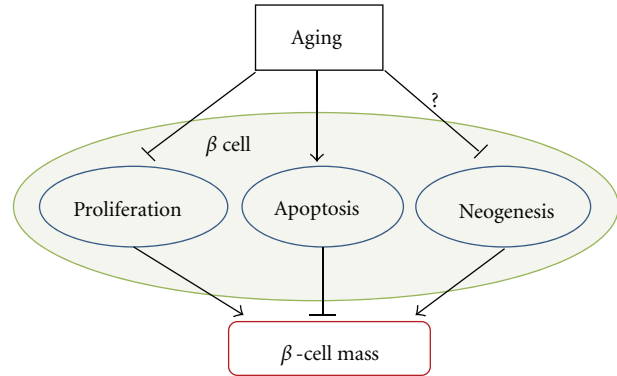


FIGURE 2: Factors that control β -cell mass in aging. β cell proliferation, apoptosis, and islet neogenesis are three major factors that control β -cell mass. Through differential regulation of these factors, aging modulates β -cell mass, and subsequently insulin secretion.

mice [50]. Although research has shown that with age there is an increased deposition of amylin in the islets of diabetic individuals but not nondiabetic individuals [2], it is still too early to rule out the potential influence of aging on amylin aggregation.

β -cell mass normally grows well into adulthood to provide increased insulin secretion capacity to match the greater insulin requirements of maturity [51]. β -cell mass can slowly expand in adult rodents in response to increased insulin requirements or during pregnancy [52]. Several mechanisms have been invoked to explain adult β -cell mass expansion, including neogenesis from pancreatic ducts or hematopoietic tissues [53], replication of specialized β -cell progenitors, and self-renewal by β cell [54, 55]. β -cell proliferation and the capacity of β cell to regenerate declines with age in mice [56]. Basal β -cell proliferation is severely decreased with advanced age. The rate of β -cell proliferation gradually declines with aging in rats to a steady state by 7 months of age [13].

Young mice respond to high-fat diet by increasing β -cell mass and proliferation and maintaining normal blood glucose levels. Old mice, in contrast, do not display any increases in β -cell mass or β -cell proliferation in response to high-fat diet and become diabetic. There is a four-fold increase in β cell proliferation in young mice after the administration of streptozotocin (STZ), a chemical that is toxic to the β cells in mammals and normally is used for inducing insulin secretion deficiency models in rodents, whereas no changes are observed in older mice [56]. Similarly, STZ stimulated β -cell replication in young mice but had little effect in old mice [57]. Partial pancreatectomy greatly stimulated β -cell proliferation in young mice but failed to increase β -cell replication in old mice [57].

p16^{Ink4a} is an effector of senescence [58] and a potent inhibitor of proliferative kinase cyclin-dependent kinase 4 (Cdk4) [59], which is essential for pancreatic β cell proliferation in adult mammals [60, 61]. Islets *in vivo* exhibit increased p16^{Ink4a} expression with age in rodents and humans [62]. Another cyclin-dependent kinase, Cdk6, is not expressed in mouse islets but is very effective in driving

β cell replication in human islets [63]. Ablation of p16 leads to improved β -cell function with age [64]. Lack of Cdk4 expression in mice leads to insulin-deficient diabetes [65]. To perform their kinase activity, Cdks bind some kinds of regulatory proteins called cyclins. Without cyclins, Cdks have little kinase activity. Mouse β cells express 3 D-cyclins, termed cyclin D1, D2, and D3; D2 is the most abundant one whereas D3 is nearly undetectable [66]. Prenatal islet development occurred normally in cyclin D2^{-/-} or cyclin D1^{+/-} D2^{-/-} mice. However, β -cell proliferation, adult mass, and glucose tolerance were decreased in adult cyclin D2^{-/-} mice, causing glucose intolerance that progressed to diabetes by 12 months of age. Although cyclin D1^{+/-} mice never developed diabetes, life-threatening diabetes developed in 3-month-old cyclin D1^{+/-} D2^{-/-} mice as β -cell mass decreased after birth. Thus, cyclins D2 and D1 were essential for β cell expansion in adult mice [66]. In contrast, in the human β cell, cyclin D3 is highly expressed, whereas cyclin D1 and D2 levels are much lower. Overexpression of cyclin D3 in isolated human islets, especially in combination with Cdk6, induced the greatest increase in β -cell proliferation when compared with over-expression of other cyclins [63]. Although cell cycle proteins play crucial roles in the proliferation of pancreatic β cells, less is known about the effects of aging on the expression or/and activity of cyclins and Cdks; therefore, studies in this area may contribute to a better understanding of the relationship between aging and loss of β -cell mass.

2.2.2. Islet Neogenesis. β cell proliferation is self-replication of the β cell, whereas islet neogenesis is the differentiation of progenitor cell or transdifferentiation of pancreatic non- β cells to β cells. Neogenesis of islets occurring during normal embryonic development and in very early postnatal life can lead to β -cell mass expansion. In addition to fetal [67] and neonatal [68] periods, β cell neogenesis has been shown to be important in increasing β -cell mass in the adult during periods of increased insulin demand such as obesity [42] and pregnancy [69]. Rosenberg et al. developed a model for islet neogenesis in adult mammalian pancreas in the 1980s, which showed that pancreatic ductal cells can be induced to differentiate into normal functioning adult endocrine cells [70]. β -cell neogenesis may occur through two pathways, stem/progenitor cell activation and transdifferentiation of adult pancreatic cells [71]. Islet neogenesis-associated protein (INGAP), found in pancreatic exocrine secretions, appears to stimulate the growth and proliferation of duct cells and their differentiation into endocrine cells [72–74]. INGAP and a bioactive 15 amino acid synthetic peptide (INGAP peptide) are inducers of islet neogenesis in a human islet system [73]. Other stimuli have been demonstrated to exert neogenic effects on the endocrine pancreas. The combination of epidermal growth factor (EGF) and gastrin has been shown to stimulate islet neogenesis in both animal and human studies [75]. Glucagon-like peptide 1 (GLP-1), an incretin from enteroendocrine L cells of the intestine, has been shown to induce islet neogenesis in rodents [76]. Although the potential for β -cell replication appears to decline substantially with age as evidenced by decreased PDX

expression, the rate of islet neogenesis (expressed as percentage of insulin positive duct cells) is not affected by aging in humans [44].

In summary, various aspects of β -cell mass and function decline as a feature of age, thus contributing to the age-associated defects in insulin secretion. This defect when superimposed for an increased need for insulin, could contribute to impaired glucose homeostasis, glucose intolerance and diabetes. In the subsequent section, we will discuss and review literature on age-associated deterioration of insulin action.

3. Aging and Insulin Action

Insulin action is the ability of insulin to bind to its receptors located on tissues including muscle, liver, and adipose tissue and initiate signaling effects. The net physiological metabolic effects that result from insulin signaling include (a) regulation of glucose homeostasis through a decrease in hepatic glucose output (via decreased gluconeogenesis and glycogenolysis) and increase in glucose uptake, primarily into striated muscle and adipose tissue as well as (b) increase in lipid synthesis in fat cells, and attenuation of release of free fatty acid from triglycerides in fat. Insulin resistance results when normal circulating concentrations of the hormone are insufficient to regulate these processes appropriately.

3.1. Visceral Fat. It is well documented that aging is associated with a decline of insulin action [77–79]. The decline in insulin action with age is thought to contribute to the high prevalence of impaired glucose tolerance and Type 2 diabetes among the elderly [80, 81]. Notably, some studies support the hypothesis that the decline in insulin action in the elderly persons is related to increased abdominal fat rather than to aging per se [82]. However, other studies suggested that age-associated insulin resistance may not be explained solely by concomitant abdominal obesity [83]. Abdominal fat (also called visceral fat, VF), which is located in and around the viscera, has been demonstrated to be strongly related to many health conditions, including CVD, insulin resistance, and T2D [84]. Several cross-sectional studies suggest that visceral fat increases throughout the lifespan in men and women of all ages and race, independent of increases in body weight [85–88]. Many factors contribute to the increased VF seen with aging such as physiologic decline in GH/IGF-1 axis, decrease in sex steroids as well as sedentary life style [89]. The mechanisms how VF links to the metabolic syndrome are still not entirely clear, but it has been suggested to involve its anatomical location, leading to a “portal” effect of greater free fatty acids (FFAs) and glycerol release [90]. Gabriely et al. showed that extraction of VF from 20-month-old Fischer 344 Brown Norway (FBN) rats was sufficient to restore peripheral and hepatic insulin action to the levels of young rats. Moreover, removal of VF in Zucker Diabetic Fatty (ZDF) rats prevented the progressive decrease in insulin action and delayed the onset of diabetes, but VF extraction did not alter plasma free fatty acid levels [91]. Borst et al. reported that VF removal in Sprague Dawley

(SD) rats tended to improve glucose tolerance and lowered some pro-inflammatory adipokines in serum; these animals displayed increased insulin-stimulated glucose transport in excised soleus and digitorum longus muscles as compared to control group. These studies provide verification that VF is a potent modulator of both hepatic and peripheral insulin action [92]. Calorie restriction (CR) extends life span and retards age-related chronic diseases in a variety of species, including rats, mice, fish, flies, worms, and yeast [93]. Our studies have shown that a reduction in fat mass, specifically VF, may be one of the possible underlying mechanisms of the antiaging effect of caloric restriction [94].

Nowadays, adipose tissue is recognized as an active metabolic-endocrine organ, and obesity is considered as a low-grade inflammatory condition strongly linked to adverse metabolic outcomes. A putative key link between increasing fat mass and obesity-related complications, including insulin resistance, is a chronic low-grade inflammatory state within adipose tissue, related to infiltration by macrophages [95]. We and others have shown that VF depots display a unique profile of inflammatory mediators compared to subcutaneous adipose tissue, including the clearly higher expression levels of macrophage migration inhibitory factor (MIF) and chemokine receptor 2 in VF [96, 97]. Other studies also suggested that VF is a stronger risk factor for metabolic disorders and mortality than subcutaneous fat [82, 98]. MIF, a proinflammatory cytokine, can activate nuclear factor (NF)- κ B signaling but directly inhibits the function of p53 [99, 100]. MIF knockout mice live longer than the control mice [101]. The possible explanation is aging is associated with inflammation and inflammation could accelerate aging process, whereas lack of MIF could downregulate NF- κ B, mediated inflammatory signaling, which will subsequently mitigate the process of aging [102]. Macrophages are considered to be a significant source for many fat-derived proinflammatory cytokines, and the percentage of macrophages in fat has been shown to increase in obesity [103]. Interestingly, our study suggested that the percentage of macrophages in the stromal vascular cell fraction from both visceral and subcutaneous fat increased with age regardless of obesity status [104]. Taken together, increase of VF is a hallmark of aging and a source of increased chronic inflammation. Inflammation could accelerate the aging process [105] and eventually lead to the metabolic dysfunction. Breaking this vicious cycle by decreasing the VF will be a potential therapeutic method for treating metabolic and related diseases (Figure 3).

3.2. Lipids and Insulin Resistance. Lack of or resistance to insulin leads to two metabolic crises: a marked increase in the rate of lipolysis in adipose tissue and activation of hepatic gluconeogenesis in spite of high plasma glucose levels. The increased rate of lipolysis increases circulating FFA levels, which, in turn, exacerbates insulin resistance in the whole body. It has been very well documented that the acute elevation of plasma FFA produces insulin resistance in both diabetic and nondiabetic individuals [106–108]. It is also shown that chronically elevated plasma FFA levels cause

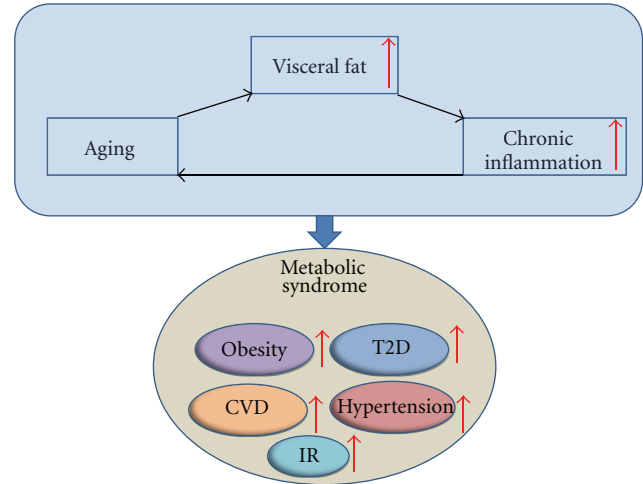


FIGURE 3: Link between Aging, visceral fat, inflammation, and metabolic syndrome. Visceral fat increases with age and the increase of visceral fat induces inflammation. Inflammation accelerates the process of aging. The vicious cycle of aging, visceral fat and inflammation increases the risk of metabolic diseases such as obesity, IR, T2D, CVD, and hypertension, CVD: cardiovascular disease, IR: insulin resistance, T2D: type 2 diabetes.

insulin resistance, and lowering elevated plasma FFA levels overnight normalizes insulin sensitivity in obese nondiabetic subjects and significantly improves insulin sensitivity in obese diabetic patients [109]. The mechanisms by which elevated levels of FFA produce insulin resistance have not been fully understood. However, studies have shown that increasing plasma FFAs acutely decreases insulin-stimulated glucose uptake and glycogen synthesis in human [110]. It is also reported that increase of FFA level in human inhibits PI3 kinase activity in skeletal muscle [111], suggesting the impairment of insulin signaling by FFA. Itani et al. pointed out that, with the infusion of FFA, increased accumulation of diacylglycerol (DAG) and protein kinase C (PKC) activity in muscle contribute to the impairment of insulin signaling [112], potentially through activation of NF- κ B [112]. The steatotic liver is also resistant to insulin in terms of inhibition of hepatic glucose production and stimulation of glycogen synthesis. The high FFA levels may be the unifying mechanism that accounts for the insulin resistance in obesity, type 2 diabetes, lipodystrophy, and aging [113]. We and others have shown that the circulating FFA levels are significant higher in 9- to 20-month-old SD rats compared to 3-month old, demonstrating that circulating FFA increases with age [113].

3.3. Aging and Central Insulin Resistance. Although peripheral insulin resistance is a hallmark of the development of T2D, more recent evidence has shown that insulin resistance also exists in central nervous system (CNS), and that central insulin action plays an important role in regulating whole body glucose metabolism. Like peripheral tissues, molecules in insulin signaling such as insulin receptor (IR), insulin receptor substrates (IRS), and phosphatidylinositol 3-kinase (PI3K) are universally expressed in the brain, indicating

a potential role of insulin signaling in the brain. Koch et al. showed that mice lacking IR in CNS exhibit significantly more severe impairment of peripheral glucose homeostasis than mice lacking IR in the peripheral tissues [114]. Gelling et al. has shown that central infusion of PI3K inhibitor attenuated insulin-induced glucose lowering by 35%–40% in both acute and chronic insulin treatment paradigms, while hypothalamic overexpression of either IRS-2, a upstream kinase of PI3K, or protein kinase B (PKB/Akt), a key downstream mediator of PI3K action, enhanced the glycemic response to insulin by 2 folds in STZ-induced diabetic rats, suggesting that hypothalamic insulin signaling is an important determinant of the response to insulin in the management of uncontrolled diabetes [115]. Interestingly, an increasing body of evidence shows a link between diabetes and AD, a neurodegenerative disorder and the most common form of dementia. It has been reported that patients with T2D increase the prevalence of AD by two-to-three folds [116], and insulin levels and insulin activity in the central nervous system are reduced in AD [117]. Studies in human subjects show that both peripheral and central administration of insulin improves memory in AD patients [118–121], suggesting impairment of insulin signaling in the brain as a risk factor of neurodegenerative disorders, and restoration of insulin signaling could be a potential therapy for AD. This brings an interesting question whether aging is also associated with central insulin resistance. Fernandes et al. demonstrated that the protein levels of elements in the insulin signaling pathway such as IRs and SRC homology adaptor protein (SHC) did not change significantly in the forebrain cortex and cerebellum of rats aged 1 d to 60 wk. However, insulin induced tyrosine phosphorylation of IR and SHC, and the association of SHC/growth factor receptor binding protein-2 (GRB2) decreased significantly in both types of tissues [122]. Other studies showed that intracerebroventricular administration of insulin was more efficient at reducing food intake and body weight in 3-month-old rats than in 8- and 24-month-old rats, indicating the development of hypothalamic insulin resistance with age in Wistar rats. Furthermore, the tyrosine phosphorylation of IR and IRS-2 and the phosphorylation of downstream target genes such as Foxo1 and p70S6K declined, whereas serine phosphorylation of IR and IRS-2 increased with age in rat hypothalamus [123].

Aging-associated increase in central and peripheral insulin resistance could contribute to both diabetes and AD. The field of central insulin resistance and its role in the development of neurodegenerative disorders and the control of whole body glucose homeostasis is complicated and further studies are needed to fully understand the underlying mechanisms. For a detailed review of the insulin signaling in the brain, we refer the readers to the following reviews [9, 124–126].

4. Aging and Whole Body Glucose Homeostasis

It is clearly established that the risk for impaired glucose tolerance and diabetes increase with age in rodents and

humans. The specific factors such as increased VF and circulating FFA that contribute to impaired insulin action and various defects in β -cell mass/function have been highlighted in the previous section. However, the integrated whole body glucose homeostasis is complex with various age-related parameters playing a crucial role on both aspects of glucose homeostasis, namely, insulin action and insulin secretion. Leptin, a hormone secreted from adipose tissue, plays a key role in energy intake and expenditure. Deficiency of leptin and its receptor leads to severe obesity, insulin resistance, and diabetes in rodents and humans. Resistant to the effects of leptin, termed leptin resistance, is seen in obesity and aging. Leptin resistance associated with aging [127–129] and decline in growth hormone (GH)/insulin-like growth factor (IGF)-1 axis [89] could play a key role in the alterations of glucose homeostasis in aging. Accumulating evidence suggests that endoplasmic reticulum (ER) stress plays a role in the pathogenesis of diabetes, contributing to both pancreatic β -cell function and peripheral insulin resistance [130]. It has been reported that aging is related to increase in proapoptotic markers with ER stress in multiple tissues, including lung, liver, kidney, and brain [131, 132]. In the past decade, a family of nicotinamide adenine dinucleotide- (NAD-) dependent protein deacetylases, termed sirtuins, have been shown to contribute to longevity. Sirtuins slow aging in worms, fruit flies, and mice [133]. Interestingly, overexpression of sirtuins or treat with activators of sirtuins, such as resveratrol protect against metabolic decline in aging, increases insulin sensitivity, increases insulin secretion, improves life quality, and extends lifespan [11, 117, 133]. In addition to the above-mentioned variables, aging-associated sedentary life style and diminished physical activity may be important factors for age-related changes of glucose homeostasis. Research has shown that healthy elderly with greater degrees of physical fitness have better glucose tolerance and lower level of insulin resistance than less active old people [134]. In addition, aging is associated with defects in the balance of insulin secretion and insulin action (demands). In the young, a hyperbolic relationship exists between insulin secretion and insulin sensitivity, whereby pancreatic β cell compensates for changes in whole-body insulin sensitivity through a proportionate increase in insulin secretion [116]. Our data shows that compared to younger animals, when challenged with a prolonged hyperglycemic stimuli older animals are unable to maintain the insulin secretion proportional to the degree of resistance [115].

Whole body glucose homeostasis is a complex balance of glucose production and utilization by different tissues. Food intake and hepatic glucose production are the two sources of glucose production, while skeletal muscle contributes to the majority of the glucose uptake and utilization. Utilizing tracer technology, it is possible to differentiate between the effects on glucose production (liver) and glucose utilization (primarily the muscle). Hepatic glucose production (HGP) plays crucial roles in glucose homeostasis, both in the fasting and postprandial states. In contrast to rodents, where there is an increase in HGP in age, there are no differences in either the basal hepatic glucose production or the dose-response curve of its

suppression by insulin between young and old individuals [135]. The European Group for the Study of Insulin Resistance reported that hepatic glucose production does not increase with age, when adjusted for lean body mass [136]. Furthermore, hepatic glucose output has not been shown to be increased in elderly patients with T2D [137]. Thus, hepatic insulin resistance does not seem play a significant role in decreased glucose tolerance of elderly people [138].

As mentioned earlier, skeletal muscle is the major source of glucose utilization. Glucose is transported into the cells by glucose transporters. Through anaerobic and aerobic pathways, glucose is broken down to generate energy. GLUT4 is the major glucose transporter in skeletal muscle responsible for insulin-stimulated glucose uptake. Muscle GLUT4 protein level is not altered in obesity and T2D, however, its expression levels decline with age, and are related to insulin sensitivity in normal controls [139]. European Group for the Study of Insulin Resistance demonstrated that glucose uptake is not altered as a function of aging per se but is secondary to increased body fat accumulation [140]. Moreover, the decrease of lean body mass [141] and contractile strength with age are other factors that contribute to the reduction in insulin stimulated glucose uptake. The above factors, along with changes in body composition, accumulation of VF, and increase in circulating FFA levels, contributes to the decreased glucose uptake with age.

5. Conclusion and Perspective

Glucose intolerance, insulin resistance, and T2D associated with aging are leading causes of morbidity and mortality through its multiple complications as well as increases in the risk for multiple other age-related diseases such as cancer, stroke, cardiovascular diseases, Parkinson's disease, and AD [6]. Though various factors that contribute to the changes in glucose homeostasis are relatively well characterized, there are still areas that are not yet fully elucidated such as the roles of aging on β -cell mass and function, and the cross-talk between central and peripheral insulin action. A comprehensive understanding of all the defects that impair glucose homeostasis in the elderly will lead to development of appropriate, novel treatments that will substantially improve quality of life and over all life span.

Abbreviations

AD:	Alzheimer's disease
Cdk:	Cyclin-dependent kinase
CNS:	Central nervous system
CR:	Calorie restriction
CVD:	Cardiovascular disease
DAG:	Diacylglycerol
ER:	Endoplasmic reticulum
FFA:	Free fatty acid
GCK:	Glucokinase
GH:	Growth hormone
GLP:	Glucagon-like peptide
GLUT:	Glucose transporter

GPDH:	Glycerophosphate dehydrogenase
GRB2:	Growth factor receptor binding protein-2
GSIS:	Glucose stimulated insulin secretion
HGP:	Hepatic Glucose Production
IAPP:	Islet amyloid polypeptide
hIAPP:	Human IAPP
IGF:	Insulin-like growth factor
INGAP:	Islet neogenesis-associated protein
IR:	Insulin receptor
IRS:	Insulin receptor substrate
K _{ATP} :	ATP-sensitive K ⁺
LDCV:	large dense core vesicles
MIF:	Macrophage migration inhibitory factor
NAD:	Nicotinamide adenine dinucleotide
NAFLD:	Nonalcoholic fatty liver disease
NF:	Nuclear factor
PI3K:	Phosphatidylinositol 3-kinase
PKB/Akt:	Protein kinase B
PKC:	Protein kinase C
SD:	Sprague Dawley
SHC:	SRC homology adaptor protein
SNAP-25:	Synaptosomal-associated protein of 25 kDa
SNARE:	Soluble N-ethylmaleimide sensitive factor attachment protein receptor
STZ:	Streptozotocin
T2D:	Type 2 diabetes
TCA:	Tri-carboxylic acid
VAMP:	Vesicle-associated membrane protein
VF:	Visceral fat
ZDF:	Zucker Diabetic Fatty.

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Review Article

Interpretation of Hormone Levels in Older Patients: Points for Consideration

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Blood hormone and tumor marker concentrations are usually determined by immunochemical methods which are based on a unique reaction between antigen and assay capture antibody. Despite the speed and simplicity of assays performance on automatic immunochemistry platforms, the interpretation of final results requires a deep knowledge of method fallibility. General lack of immunoassays standardization, presence of cross-reacting substances in patient's sample, limitation of free hormones measurement due to abnormal analyte binding protein concentrations, assay interferences due to patient's autoantibodies, and heterophilic antibodies, as well as proper interpretation of very low- and very high-sample analyte levels, are the main points discussed in respect to hormones and tumor markers measurement in geriatric population.

1. Introduction

Aging process is associated with physiological changes in function of almost every organ and system, including the endocrine system. The function of endocrine glands function declines progressively with age. For example, dehydroepiandrosterone sulphate (DHEAS) concentration is about 10–20% of maximum in patients at the age of 70–80 years [1]. The activity of the growth hormone/IGF axis also declines steadily, and a sudden cessation of the function of some elements of the hormonal system is well documented [2–5]. Negative feedback loop is the common regulatory mechanism within endocrine system. Thus, markedly decreased or markedly increased blood hormones concentrations can be measured in elderly patients. Multiple diseases, which frequently entail polytherapy with the use of multiple drugs, influence the levels of hormones *in vivo* and the measurement biomarkers as well as drugs concentration by immunochemistry *in vitro* [6]. The coexistence of multiple diseases may cause secondary changes in hormone levels as it is the case with the level of thyroid-stimulating hormone (TSH) in nonthyroidal diseases [7–9]. Moreover, in an elderly population, the presence of autoantibodies due to autoimmune or

chronic diseases is more frequent than it is in the younger population. The presence of autoantibodies in the plasma or serum, like rheumatoid factors (RFs), may affect the determination of many hormones by immunochemistry, or if directed against a specific molecule, such as prolactin or troponin, they may mislead the medical decision based on hormone measurement [10–13]. In addition, changes in the serum level of specific and nonspecific hormone-binding protein, especially albumin, frequently observed in geriatric population, greatly influence the measurement of free hormones [14]. Furthermore, the fallibility and limitation of immunochemical methods of hormone measurements may lead to clinically misleading interpretation of laboratory results of hormone concentration in elderly population.

Hormones, proteins, peptides, tumor markers, and drugs are routinely measured using automated immunochemistry platforms. Immunochemistry methods are based on the reaction between an antigen and an antibody; both competitive and noncompetitive method formats are used. The reaction between antigen and antibody is very specific due to unique properties and stereochemistry of epitope on the antigen and paratope on the antibody. Although the analytical procedure for measuring hormones is very simple and easy

to perform, the interpretation of results requires not only medical knowledge but also deep knowledge of immunochemistry limitations. This is especially true when hormone measurements are performed in the serum of the patient at an extremely advanced age, the patient with multiple or chronic disease, and the patient on multiple drugs therapy.

For all the laboratory determinations, the preanalytical phase of a diagnostic procedure contributes the most to the total laboratory error of measurement, regardless of the patient's age. The type of anticoagulants used, the presence of hemolysis, lipemia, hyperbilirubinemia, and paraproteinemia are well-known factors, influencing the measurement of biochemical markers, including hormones. The observed bias due to hemolysis may be negative, as it is the case with cortisol, parathyroid hormone (PTH), and insulin measurement, or positive as it is the case with troponin I determination [15, 16]; or susceptibility to interference by hemolysis is different, as it was shown for cardiac troponin I and troponin T measured by current immunoassays [17]. Some hormones are very labile *ex vivo*, and negative bias due to proteolysis is frequently seen in the measurement of peptides, such as adrenocorticotropic hormones (ACTH), insulin, osteocalcin, C-peptide, and PTH [18, 19]. Although the concentrations of most hormones are measured directly in the serum or plasma, it is necessary for many hormones to treat the blood sample specifically before analysis, as in the case of gastrointestinal peptides measurement [20, 21].

Apart from the aforementioned pre non-specific analytical problems, there are many pitfalls which may occur during the analytical phase of hormones determination by immunochemical methods, which are known by the laboratory personnel but frequently unknown by physicians. For a proper interpretation of the hormone concentration results, the comparison of the results with appropriate reference intervals coupled with good clinical knowledge is necessary. In case of discrepancy between the laboratory data and the clinical picture of the patient, repeated analytical measurements are usually requested. However, in the case of hormones and tumor markers, repeated measurements of the analyte by immunochemistry in questionable patients' samples give concentration results that do not always meet clinical expectations. To avoid such a situation, it is important for clinicians to know and to understand the limitation and fallibility of immunochemical methods in order to protect the patient from misdiagnosis. This is extremely important for every patient, but it must be stressed that in samples from geriatric patients, the presence of various drugs and their metabolites, the presence of autoantibodies and other inducible antibodies, and low albumin level, as well as disturbances in specific and nonspecific hormone-binding protein levels, are frequently observed. In addition, tumor marker measurements are much more frequently requested in older patients as compared to other age groups, extremely high level of some proteins can be expected as well. On the other hand, after surgery of the endocrine gland due to cancer, or during suppressive therapy, the measurement of very low level of some hormones is important for clinical management of a geriatric patient. Thus, for proper interpretation of the laboratory results of hormones and tumor markers determination, it is

advisable for physicians to become familiar with most important immunochemistry issues, so that they could answer the following questions: (a) what is being measured by a given immunoassay? (b) how accurate are low and high concentrations of hormone/tumor marker measured? (c) how do binding proteins affect hormone measurement? (d) how do autoantibodies; heterophilic and anti-animal antibodies interfere with the measurement of hormone/tumor marker?

1.1. What Is Being Measured by the Immunoassay? Different chemical molecules, such as protein, peptides, biogenic amines, steroids, and drugs, can be measured by immunochemical methods. As for any other methods, standardization of immunochemical methods is necessary to ensure accuracy of a measurement and comparability of results between different assays. However, most of the immunoassays lack proper standardization. Although the primary standards are available for small molecules (amines, steroids, and drugs), the lack of commutability between primary or secondary standards and the patient's samples due to matrix effect make the standardization process a very difficult task. On the other hand, many hormones of clinical interest are present in the blood in heterogeneous forms (growth hormone, prolactin, gonadotropins, TSH, and gastrin) [22–25] or in monomeric and dimeric forms (insulin) [26, 27]. For heterogeneous molecules, the exact definitions of the substance intended to be measured by immunoassay should always be specified by manufacturers because depending on the specificity of antibodies used in immunochemical methods, different forms of protein can be measured [28]. In addition, plasma samples contain a vast variety of molecules, and there is always a possibility that a chemical structure recognized by an immunoassay capture antibody can be found not only on the molecule of interest, but also on “cross-reacting” substances. Heterogeneity of proteins and their structural similarity and the presence of cross-reacting substances in patients' samples can be a source of false positive (competitive methods) or false negative (noncompetitive methods) results [28–31]. In case of heterogenous proteins, harmonization can be used to improve comparability of methods. The purpose of the harmonization process is to obtain similar results for the analyte measured by different “harmonized” immunoassays calibrated with the use of the same calibrator. It has to be stressed out that harmonization of methods is not equivalent to method standardization.

Immunochemical methods for the measurement of the same analyte may differ with respect to reagent antibodies and to a different standard for calibration. As a consequence, the results of the concentration of hormones and tumor markers obtained by different assay or immunochemistry platforms are often not comparable. Thus, two issues are of great importance: firstly, the knowledge of the molecule that is being measured by immunoassay; secondly, the mandatory use of the method-dependent reference intervals established by the laboratory. Taking into account the lack of immunoassays standardization, heterogeneity of many peptides and protein, structural similarities of steroids and their metabolites, as well as capture antibody specificity, the request for

the hormone or tumor marker measurement by two laboratories using different immunoassays should be avoided.

1.2. How Accurate Are Low and High Concentrations of the Hormone/Tumor Marker Being Measured? Analytical sensitivity is an important issue for those analytes for which low concentrations in the patient's sample are diagnostically important as it can be observed in geriatric population in case of C-reactive protein (CRP), estrogen, TSH, and troponin measurement [32–35]. Interpretation of very low concentrations of some analytes requires understanding the analytical sensitivity which, in the simplest way, can be defined as the lowest hormone concentration that can be determined by a given method. From the clinical point of view, it is not enough to accept the limit of the absence of the analyte in the sample based on a repeated zero standard measurement and taking the mean value plus two or three standard deviations. Since each analytical measurement is burdened with error, it is important to know what is the limit of analyte quantification (functional sensitivity of the method). For proper interpretation of a low analyte level, it is necessary to know the concentration value that is measured with certain, predefined precision, usually 10–20%. Many methods are not sensitive enough to measure, for example, low estrogen level (characteristic for pediatric population, men and postmenopausal women) or TSH in geriatric population with nonthyroidal illnesses. It should be noted that a laboratory cannot determine the concentration of any analyte below the functional sensitivity with acceptable precision and accuracy, and any approximation of standard curve below the value of concentration determining functional sensitivity is the unacceptable analytical practice. It is especially important when C-reactive protein (CRP) is not measured by highly sensitive CRP method (hsCRP), and clinical judgment is made on the basis of the CRP level as a prognostic factor in a geriatric patient. The measurement of CRP by two immunoassays differing in analytical sensitivity and using the results interchangeably, that is, both as an inflammatory marker and a prognostic marker of future cardiovascular events should never be done. Another example showing the importance of functional sensitivity in geriatric population is troponin measurement, since a very small increase in its concentration may have serious clinical consequences [36]. Each laboratory that measures troponin level should have functional sensitivity established under routine conditions, and physicians should be aware of such concentration value in order to avoid patient misdiagnosis.

Measurement of very high or extremely high concentration of hormones and tumor markers is a great challenge for laboratory staff, since disagreement between the clinical picture of the patient and the laboratory result is sometimes noted. This is especially true for a geriatric population because the frequency of oncologic diseases of different origin increases with age. In immunochemical noncompetitive methods, unlike in other analytical methods, a high-dose effect (hook effect) may occur. In such methods, antigen is linked with two assay antibodies (solid-phase capture antibody and signal antibody) forming a so-called “sandwich”, and the proportionality between the assay signal and analyte

concentration is seen. However, the enormous amount of the analyte in the patient's sample blocks both assay capture and labeled antibodies, which does not allow for the formation of a typical “sandwich” [28]; and the linear relationship between the magnitude of the assay signal and the concentration of analyte no longer exists. As a consequence, assay signal is descending. This means that the same assay signal is obtained for the low and the very high analyte concentration. As a result, falsely low, frequently normal, concentration of the analyte is measured [37]. This effect occurs more frequently in homogenous noncompetitive assays compared to heterogeneous noncompetitive assays. If hook effect is suspected, each laboratory performs the dilution test until stable assay signal is obtained. Erroneous results due to hook effect can be observed, among others, for carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), prostate-specific antigen (PSA), prolactin, thyroglobulin, and cancer-antigen CA-125 [38–44]. Requesting the tumor marker measurement, either for the patient diagnosis or for monitoring therapy, and suspecting the hook effect in an oncologic patient are of great importance because only the abnormal concentration results (outside reference intervals) alert laboratory personnel and physicians; the results within normal range or below cutoff points rarely undergo additional laboratory procedure, unless there is a disagreement between the laboratory result and the patient clinical condition.

1.3. How Do Binding Proteins Affect Hormone Measurement?

In the geriatric population, the results of hormones measurement should be interpreted with caution as age-related decline in concentration is characteristic not only for specific binding proteins, such as insulin-like growth factor-binding protein 3 (IGFBP-3), but also for nonspecific binding proteins, such as albumin [45–47]. It may be expected that decreased serum albumin concentration is related to aging; however, it is caused more often by chronic malnutrition than by aging itself [48]. Prolonged decrease in albumin concentration is characteristic of liver and kidney disease, congestive heart disease, and protein-losing enteropathies. Inflammation, frequently seen in elderly population, is another cause of low albumin level, as albumin is a negative acute-phase protein [49]. Changes in specific levels of hormone-binding proteins influence the discrete equilibrium which exists between bound and free hormone fraction. Changes in nonspecific binding protein concentration do not only influence the balance between free and bound fraction of hormone but also have a great impact on the plasma level of many biochemical parameters as these proteins bind and release different ligands, changing the sample matrix. This is especially true for albumin, which is an universal carrier for drugs, metals, fatty acids, vitamins, steroids, minerals, and hormones; binding/releasing different ligands strongly depends on pathological condition [50–53].

A good example of the effect of binding proteins on hormone determination is the estimation of free-thyroid hormone levels (FT4 and FT3) in elderly population. The FT4 plasma concentration depends on the binding capacity of thyroxine-binding globulin concentration (TBG) as well as albumin and prealbumin. Depending on the immunoassay

format, either false positive (competitive methods) or false negative (noncompetitive methods) results of free hormone measurement can be obtained as an effect of binding proteins interference [28]. Interpretation of FT4 is also difficult in a female patient on estrogen therapy because estrogen excess is associated with a rise of TBG concentration as well as in a patient on androgen therapy in whom a marked decreased TBG level is observed [54].

In geriatric patients treated with heparin (including low-molecular-weight heparin), a misleading diagnosis can affect the patients' safety due to falsely elevated FT4. The concentration of FT4 in such patients depends on the time that elapsed between heparin administration and blood sampling as well as the time that elapsed between the collection of the blood and performing immunoassay measurement [55]. Free fatty acids released due to *in vitro* lipolysis displace T4 from its binding protein complexes. Thus, a false increase in FT4 concentration is seen. In relation to this, in the interpretation of hormone level in the plasma in both free and bound fraction, a low albumin level should always be taken into consideration.

Lower serum albumin level frequently observed in geriatric population is also associated with the decrease in maximum binding capacity of drugs, which is significant during polytherapy. As a consequence, free-drug concentrations in the plasma are increased [56]. Common drugs used in the geriatric population strongly bind to plasma protein (tricyclic antidepressants, psychotropic medications, benzodiazepines, phenytoin and warfarin). Hence, any disturbance in binding proteins influences the plasma drug concentration measured by immunochemistry.

1.4. How Do Autoantibodies, Heterophilic, and Anti-Animal Antibodies Interfere with Hormone/Tumor Markers Measurement? Common health problems encountered in the geriatric population include various chronic inflammatory diseases such as rheumatoid arthritis, pneumonia, and systemic lupus erythematosus (SLE) [57–60]. In such conditions, the presence of different autoantibodies in the blood is observed, and the prevalence of increased concentration of autoantibodies increases with age [61, 62]. Changes in the immune system are associated with a high incidence of antibodies such as rheumatoid factors (RFs), antinuclear antibodies (ANA), antibodies against single-strand DNA (ssDNA), antibodies against double-strand DNA (dsDNA), antiphospholipid antibodies, antibodies against hormones (i.e., antibodies against insulin), and anti-IGFBP2 antibodies [61]. In plasma samples, they represent a large variation in titer at different periods of time, depending on the patient's disease stage. In the geriatric population, the presence of organ and nonorgan specific autoantibodies is common; and frequently, no visible symptoms of disease are seen [61]. Autoimmune thyroid disease is a frequent pathology in geriatric patients which includes Graves' disease, Hashimoto's thyroiditis, hyperthyroidism, and hypothyroidism. Therefore, increased levels of autoantibodies against thyroglobulin (anti-Tg), autoantibodies against thyrotropin receptor (TRAB), and antithyroid peroxidase antibodies (anti-TPO) are also noted [63].

From the analytical point of view, different problems have to be considered if it is necessary to measure the concentration of protein against which autoantibodies are present in the plasma sample or measurement of autoantibodies as an independent marker of immune disease is requested. Firstly, autoantibodies can interfere with the analyte measurement, giving erroneous results and, depending on the assay format, both underestimation (noncompetitive methods) or overestimation (competitive methods) can be observed [28]. The most typical example is the thyroglobulin concentration measurement in the presence of antithyroglobulin antibodies in a patient with differentiated thyroid carcinoma [64–67]. Secondly, if the level of autoantibodies is to be measured, final results depend, to a large extent, on the amount of endogenous self-antigen already bound to autoantibodies and frequently, no agreement between competitive and noncompetitive immunoassay formats is achieved. Thirdly, human immunoglobulin frequently forms a complex with the target protein intended to be measured, forming a so-called macroprotein. The complex of prolactin with immunoglobulin is one of the well-recognized protein macroform by endocrinologists. A complex of monomeric protein with immunoglobulin, usually IgG, may not be active *in vivo* but can be immunoreactive, so it can be detected by immunoassays [28]. It is assumed that macroprotein has no biological activity, but its half-life is much longer than the half-life of monomeric protein. Thus, macroprotein accumulates in the blood. Depending on the immunoassay format, both free and complexed forms of protein may be detected by the immunoassay or only the free form is measured but with macroprotein invisible by the assay antibody. Recently, analytical problems connecting the measurement of troponin in a sample in the presence of autoantibodies against troponin T and troponin I have been discussed in the literature [68–70]. Since the frequency of protein macroform formation increases with age, it is important to take such interference into consideration in case of disagreement between clinical picture of patient and laboratory immunoassay result.

In addition to autoantibodies against self-antigens, any inducible antibodies against different foreign antigens can be present in human serum samples. Such inducible antibodies are usually polyreactive and are directed against poorly defined foreign antigens. For analytical purposes they have been called heterophilic antibodies. The best known heterophilic antibodies are rheumatoid factors [71]. In serum or plasma samples, human anti-animal antibodies produced against antigen of animal origin, such as human anti-mouse antibodies (HAMA) or human anti-rabbit antibodies (HARA), can also be present [72–74]. Both human heterophilic and human anti-animal antibodies have properties similar to autoantibodies with respect to binding to immunoassay reagent antibodies [75]. This means that these human antibodies have the ability to interfere in an immunoassay reaction by binding to reagent assay antibodies (animal origin, usually mouse monoclonal antibody) either by binding to the epitope or by sterically blocking the access of antigen to the binding site. The type, amount, and affinity of human interfering antibodies (heterophilic or anti-animal) in

a patient's sample are usually unknown and variable [76–79]. The mechanism of heterophilic antibody interference depends on the assay format; both falsely elevated, falsely positive, and falsely decreased results can be obtained. It is impossible to predict the mode of reaction between assay reagent antibodies and interfering antibodies just as it is almost impossible to judge a priori in which patient's sample interference will occur.

In the geriatric population, interference from heterophilic antibodies is as extremely important as the variety of antibodies that are present in the blood. For example, rheumatoid factors are present in 70% of patients with rheumatoid arthritis and the occurrence of human anti-mouse antibodies as a consequence of treating the patient with mouse immunoglobulin for diagnostic and therapeutic purposes is estimated as high as 11.7% [80]. In the literature, a plethora of papers describe the unexpected interference from anti-animal antibodies, for example, in the PTH measurement in serum sample of a patient treated with murine monoclonal antibody directed toward different surface antigens of human T-cell immunoglobulin [81]. It is expected that the problem of interference from heterophilic and human anti-mouse antibodies will increase as new therapeutic approaches for cancer treatment are introduced.

2. Conclusions

Accuracy of analytical measurement of different biochemical parameters is a prerequisite for proper diagnosis and treatment monitoring of the patient. Immunochemical methods play an important role in measurement of a variety of biochemical molecules, although due to their fallibility, many limitations in measurement are noted. Immunochemistry is a very powerful analytical technique, but imperfections in analytical measurement are directly connected with unique basis of methods, general lack of standardization, and presence of many interfering substances in patients' samples. The more a patient's sample matrix differs from the normal sample matrix, the higher the probability that erroneous results will occur. In older patients, misinterpretation of immunochemistry results due to the presence of interfering endogenous substances (cross-reacting substances, abnormal hormone binding proteins, presence of autoantibodies, heterophilic antibodies, and anti-animal antibodies) in the blood is more frequent than in younger individuals. It has to be stressed, that most pitfalls in analyte measurement by immunochemistry are related to a patient's sample, and no quality control assurance program exists to protect patient from erroneous results. The only way to suspect an error in immunochemistry results is through the information obtained from physicians, where there is disagreement between laboratory results and the patient's condition. Each laboratory has procedures to look for errors in immunochemistry measurement, but the information must first come from clinicians. The more signals from physicians, the higher the possibility in avoiding immunochemistry errors in the future. In order to achieve this, the physician taking care of the geriatric population should be familiar with the limitations of immunochemistry.

Abbreviations

CRP: C-reactive protein
 FT4: Free thyroxine
 IGF: Insulin-like growth factor
 PTH: Parathyroid hormone
 RFs: Rheumatoid factors
 TBG: Thyroxine-binding globulin
 TSH: Thyroid-stimulating hormone.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Interactions between Serum Adipokines and Osteocalcin in Older Patients with Hip Fracture

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Introduction. Experiments on genetically modified animals have discovered a complex cross-regulation between adipokines (leptin, adiponectin) and osteocalcin. The relationships between these molecules in human osteoporosis are still unclear. We evaluated the hypothesis of a bidirectional link between adipokines and osteocalcin. **Materials and Methods.** In a cross-sectional study of 294 older patients with osteoporotic hip fracture, we estimated serum concentrations of leptin, adiponectin, resistin, osteocalcin, parameters of mineral metabolism, and renal function. **Results.** After adjustment for multiple potential confounders, serum osteocalcin concentration was inversely associated with resistin and positively with leptin, leptin/resistin ratio, and adiponectin/resistin ratio. In multivariate regression models, osteocalcin was an independent predictor of serum leptin, resistin, leptin/resistin, and adiponectin/resistin ratios. **Conclusions.** Our data support the bidirectional regulation between osteocalcin and adipokines, but contrary to the genetically modified animal models, in older subjects with osteoporotic hip fracture, serum osteocalcin is positively associated with leptin and inversely with resistin.

1. Introduction

Over the past two decades, it has been convincingly shown that adipose tissue is an active endocrine organ which produces a number of biologically active molecules named adipokines. More recently, the endocrine function of the skeleton and its important role in metabolic homeostasis has been revealed [1, 2]. Mainly through mouse genetic means by analysing loss-of-function models, the existence of a complex bilateral hormonal link (crosstalk) between bone and energy metabolism has been discovered [1–4]. According to the current paradigm, bone remodelling and energy metabolism are coregulated by adipocyte-derived hormones, leptin, and adiponectin, and the feedback loop between bone and energy metabolism is mediated by osteocalcin (OC), an osteoblast-specific protein. The biological importance of tight connections between adipose tissue and bone remodelling is further supported by the fact that adipocytes and osteoblasts are derived from a common mesenchymal progenitor cell [5],

leptin and adiponectin are expressed in osteoblasts [6–8] and OC in human adipocytes [9]. A crosstalk between signalling pathway regulating adipocyte and osteoblast differentiation has also been recently described [10].

Results of experimental studies on reciprocal bone-energy metabolism relationships mediated by adipokines and OC are fairly consistent. However, clinical data on the association between circulating leptin and adiponectin levels and OC are controversial. Previous human studies that have evaluated the relationship between leptin and OC yielded conflicting results, showing either no correlation [11–16], positive [17], or negative correlation [18–21]. Similarly, some studies reported a positive association between serum adiponectin and OC [22–27], whereas other studies were not able to demonstrate a significant and independent relationship [16, 20, 28–30].

Emerging evidence has shown that resistin, a peptide hormone classified as an adipokine, although in humans it is mainly produced by mononuclear cells and macrophages, is

important in regulating insulin resistance, diabetes, inflammatory processes, immunity, and bone metabolism [17, 31–34]. However, the interrelations between resistin and OC have not been characterised.

In the above-mentioned studies, such factors as serum calcium, phosphate, magnesium, 25-hydroxyvitamin D (25(OH)D), parathyroid hormone (PTH), renal status, and age, known to influence both bone metabolism and circulating adipokines, have rarely been measured and analysed. Lack of assessment of several adipokines simultaneously, difference in study populations and the dual nature of leptin's effect on the skeleton (central antiosteogenic [35] and peripheral osteogenic [5]) may also contribute to the inconsistency in human data.

There are only a few studies evaluating leptin [36, 37] and adiponectin [38] in patients with hip fracture (HF), but no research has been carried out showing the relationship between adipokines and OC in these patients. It remains to be determined whether the phenomenon of bidirectional adipokine-OC interaction is involved in human osteoporosis. Therefore, the aim of the present study was to assess in older patients with HF the association of leptin, adiponectin, and resistin, the three most widely investigated adipokines, with OC, and to examine whether OC is a significant and independent predictor of circulating adipokine levels. We also analysed the combined effect of adipokines using their ratios, since it has been suggested that metabolic functions of adipokines, especially of leptin and adiponectin, are complementary, and the leptin/adiponectin and adiponectin/resistin ratios are better clinical indicators [39–43].

2. Patients and Methods

2.1. Patients. A total of 294 consecutive older patients (≥ 60 years of age, mean age 82.1 ± 8.0 years) with low-trauma osteoporotic HF were included in this study. Data were obtained from a prospective electronic database on all adult patients with fracture of the upper femur admitted to the orthopaedic ward of The Canberra Hospital (Canberra, Australian Capital Territory, Australia), a university-affiliated tertiary care centre. Exclusion criteria were subtrochanteric and shaft fracture, age < 60 years, high trauma, and pathological HF due to primary or metastatic bone cancer, multiple myeloma, Paget disease, or primary hyperparathyroidism. Sociodemographic, anthropometric, clinical (HF type, comorbidities, complications, medication use) and laboratory data were recorded.

Informed consent was obtained from all patients or their carers. The study has approval of the local Research Ethics Committee.

2.2. Laboratory Measurements. In all patients, antecubital venous blood samples were collected after overnight fast within 48 hours of arrival at the Emergency Department. Routine haematological and biochemical assessments were performed by standardised methods on autoanalysers at the day of collection. For assays of OC, leptin, adiponectin,

and resistin serum samples were frozen in liquid nitrogen and stored at -70°C , subsequently thawed and analysed in a single batch using commercially available kits. Serum levels of OC were determined by electrochemiluminescence immunoassay (Eleclys 1010; Roche Diagnostics, IN, USA; analytical sensitivity 0.5 ng/mL, interassay coefficient of variation (CV) 2.1–3.1%, intraassay CV $< 3\%$), leptin by enzyme-linked immunosorbent assay (ELISA) method (Diagnostic System Laboratories, Webster, TX, USA; sensitivity 0.05 ng/mL, interassay CV 3.4–5.5%, intraassay CV $< 6\%$), total adiponectin and resistin by human ELISA kits (B-Bridge International, Mountain View, CA, USA; for adiponectin sensitivity 0.5 ng/mL, intraassay CV 3.2–7.3%, interassay CV $< 6\%$; for resistin sensitivity 0.03 ng/mL, interassay CV 4.5–7.2%, intraassay CV $< 5\%$). All assays were performed with kits of the same lot number.

Serum levels of 25(OH)D were determined by a radioimmunoassay kit (Dia Sorin, Stillwater, MN, USA; sensitivity 0.7 pmol/L, interassay CV 5.9–9.4%, intraassay CV $< 11.5\%$), intact PTH by solid-phase two-site chemiluminescent enzyme-linked immunometric assay on a DPC Immulite 2000 analyzer (Diagnostic Products, Los Angeles, CA, USA; sensitivity 0.07 pmol/L, interassay CV 6.2–7.0%, intraassay CV $< 6\%$). Serum calcium concentration was corrected for serum albumin. Glomerular filtration rate (eGFR) was estimated by the formula [44].

2.3. Statistical Analyses. All analyses were performed using Stata software (version 10; StataCorp, College Station, TX, USA). The summary statistics are presented as the mean \pm standard deviation for continuous variables and as the number (percentages) for categorical variables. Continuous variables with a skewed distribution were logarithmically transformed before being used in correlation analyses. The relationships between variables were examined by Pearson's linear correlation test and multivariate logistic regression analyses. $P < 0.05$ (two-sided) was considered statistically significant. To assess the potential effect of multiple comparisons and the significance of multicollinearity phenomena in multivariate regression analyses, Bonferroni's and Sidak's corrections were used and the variance inflation factor was calculated.

3. Results

3.1. Patient Characteristics. The demographic and clinical characteristics of the study patients are shown in Table 1. There were 212 (72.1%) women and 82 (27.9%) men. Women were found to be slightly older than men (82.6 ± 7.7 versus 80.6 ± 8.3 years, $P = 0.053$). The HF was of cervical type in 52% and of trochanteric in 48% of patients. The mean (\pm SD) values of serum 25(OH)D and PTH were 37.2 ± 18.0 nmol/L and 6.9 ± 5.6 pmol/L, respectively. Vitamin D deficiency (25(OH)D < 50 nmol/L) was found in 84.6% of females and 67.5% of males ($P < 0.008$) and secondary hyperparathyroidism (PTH > 6.8 pmol/L) in 39.4% and 25.3%, respectively ($P = 0.028$). The mean serum osteocalcin level was 17.2 ± 15.2 ng/mL. The serum

TABLE 1: Demographic and clinical characteristics of the study patients with hip fracture ($n = 294$).

Characteristic	
Age, years (mean \pm SD)	82.2 \pm 7.9
Females, %	72.1
Comorbidities:	
Hypertension, %	54.0
CAD, %	21.2
Previous myocardial infarction, %	5.3
Atrial fibrillation, %	13.2
History of stroke, %	14.3
TIA, %	7.4
Type 2 DM, %	18.8
Dementia, %	27.8
Parkinson's disease	4.4
CKD stage \geq 3	42.6
COPD	11.8
Malnutrition	33.8

CAD, coronary artery disease; TIA, transient ischaemic attack; DM, diabetes mellitus; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease.

osteocalcin levels were low (<14 ng/mL) in 53.3% of patients. The osteocalcin concentrations did not differ significantly with respect to gender or HF type. The main concentrations of serum leptin, adiponectin, and resistin were 18.4 ± 23.2 ng/mL, 17.5 ± 7.4 ng/mL, and 18.7 ± 10.5 ng/mL, respectively. Women had significantly higher mean serum concentrations of leptin (21.1 ± 24.3 versus 11.7 ± 18.6 ng/mL, $P = 0.002$), adiponectin (18.3 ± 7.1 versus 15.6 ± 7.6 ng/mL, $P = 0.007$), leptin/resistin ratio (1.6 ± 2.3 versus 0.8 ± 1 , $P = 0.006$), and PTH (7.4 ± 6.1 versus 5.5 ± 3.5 pmol/L, $P = 0.009$), but lower levels of 25(OH)D (35.3 ± 17.6 versus 42.4 ± 18.2 nmol/L, $P = 0.009$), phosphate (0.89 ± 0.29 versus 1.07 ± 0.75 nmol/L, $P = 0.003$), magnesium (0.76 ± 0.13 versus 0.81 ± 0.12 nmol/L, $P = 0.008$), and eGFR (62.7 ± 22 versus 71.2 ± 26.4 mL/min./1.73m², $P = 0.006$). Mean serum concentrations of resistin, leptin/adiponectin, and adiponectin/resistin ratios, osteocalcin, calcium (corrected for albumin), TSH, albumin, and haemoglobin in women and men did not differ.

Malnutrition defined as serum leptin concentration <4 ng/mL in males and <6.5 ng/mL in females [45] was observed in 33.8% of patients (equal in both sexes). The malnourished group compared to the rest of the cohort was older (83.6 ± 7.8 versus 81.2 ± 8.0 years; $P = 0.015$) and as would be expected had higher serum levels of adiponectin (19.3 ± 6.6 versus 16.5 ± 7.5 ng/mL; $P = 0.035$) and lower levels of leptin (2.9 ± 1.3 versus 26.5 ± 25.0 ng/mL; $P < 0.001$), haemoglobin (121.3 ± 16.3 versus 126.3 ± 17.4 g/L; $P = 0.021$), leptin/adiponectin (0.17 versus 2.13 ; $P < 0.001$), and leptin/resistin (0.22 versus 1.94 ; $P < 0.001$) ratios; OC levels were also lower, however, the difference did not reach

statistical significance (15.3 ± 9.7 versus 18.2 ± 17.3 ng/mL; $P = 0.117$).

Patients with cervical compared to trochanteric HF had higher serum levels of adiponectin (18.5 ± 7.3 versus 16.3 ± 7.3 ng/mL, $P = 0.019$) and resistin (20.1 ± 10.5 versus 16.9 ± 10.5 ng/mL, $P = 0.014$), lower leptin/resistin ratio (1.1 ± 1.4 versus 1.7 ± 2.6 , $P = 0.025$), and PTH concentrations (5.9 ± 3.6 versus 8.0 ± 6.9 pmol/L, $P = 0.001$), but did not differ significantly regarding other parameters.

3.2. Correlations of Adipokines with Serum Osteocalcin, Parameters of Mineral Metabolism, Renal Status, and Age. Pearson correlation analysis performed with log-transformed variables revealed that leptin correlated positively with osteocalcin ($r = 0.123$, $P = 0.038$), BMI ($r = 0.210$, $P = 0.001$), and haemoglobin ($r = 0.188$, $P = 0.001$) and inversely with adiponectin ($r = -0.178$, $P = 0.005$), phosphate ($r = -0.161$, $P = 0.007$), and age ($r = -0.154$, $P = 0.009$). Adiponectin correlated positively with PTH ($r = 0.193$, $P = 0.002$) and age ($r = 0.251$, $P = 0.001$) and negatively with BMI ($r = -0.170$, $P = 0.005$).

Resistin correlated positively with age ($r = 0.156$, $P = 0.013$) and negatively with serum magnesium ($r = -0.198$, $P = 0.002$) and eGFR ($r = -0.126$, $P = 0.044$). Serum osteocalcin correlated positively also with leptin/adiponectin ratio ($r = 0.129$, $P = 0.041$), leptin/resistin ratio ($r = 0.166$, $P = 0.008$), calcium ($r = 0.169$, $P = 0.004$), phosphate ($r = 0.129$, $P = 0.003$), magnesium ($r = 0.124$, $P = 0.038$), and age ($r = 0.152$, $P = 0.010$) and negatively with eGFR ($r = -0.388$, $P = 0.001$) and 25(OH)D ($r = -0.127$, $P = 0.037$).

3.3. Adipokines and Their Ratios as Independent Determinants of Serum Osteocalcin. Multiple regression analyses were performed to evaluate which individual adipokine or their ratios are independently associated with serum osteocalcin. As shown in Table 2, there was a significant positive correlation between serum log-leptin and log-osteocalcin before and after multiple adjustments. No relationship was found between log-transformed serum adiponectin and log-osteocalcin. Serum log-resistin was negatively and significantly associated with log-osteocalcin only after adjusting for age, sex, BMI, HF type, 25(OH)D, and PTH. This association remained significant after all further adjustments. In the final regression when all three adipokines were used in place of one, the independent determinants of serum osteocalcin were leptin ($P = 0.040$), resistin ($P = 0.018$), age ($P = 0.018$), and eGFR ($P < 0.001$). The model explained 39.5% of the variance in OC. Taking into account that type 2 diabetes mellitus (DM), hypertension, and other cardiovascular diseases which are common in the elderly population (Table 1) are known to be associated with dysregulation in adipokine metabolism, we further adjusted our models for these comorbidities (yes/no). These adjustments did not appreciably change the estimates for OC-leptin and OC-adiponectin associations. Neither hypertension (*per se*) nor any cardiovascular disease affected the OC-resistin relationship. However, addition of type 2 DM to the models

TABLE 2: Associations of serum leptin, adiponectin, and resistin with serum osteocalcin as dependent variable in linear regression models.

Adjustments	Leptin		Adiponectin		Resistin	
	β	<i>P</i>	β	<i>P</i>	β	<i>P</i>
Unadjusted	0.071	0.038	-0.015	0.859	-0.064	0.372
+ Age, sex, BMI	0.093	0.008	-0.087	0.329	-0.099	0.167
+ HF type	0.093	0.009	-0.084	0.350	-0.097	0.182
+ 25(OH)D	0.081	0.023	-0.126	.0162	-0.138	0.055
+ PTH	0.080	0.024	-0.138	0.129	-0.169	0.022
+ Ca, Mg, PO ₄	0.079	0.027	-0.123	0.072	-0.178	0.019
+ eGFR	0.055	0.105	-0.073	0.406	-0.162	0.026
+ Haemoglobin	0.076	0.046	-0.075	0.394	-0.162	0.026
+ Two other adipokines	0.077	0.040	-0.051	0.567	-0.173	0.018
+ Type 2 DM	0.090	0.018	-0.053	0.551	-0.119	0.126
+ Hypertension*	0.078	0.046	-0.055	0.544	-0.173	0.021
+ CVD (any)*	0.090	0.023	-0.059	0.575	-0.156	0.037
+ CVD (any)	0.099	0.014	-0.061	0.507	-0.104	0.191

Leptin, adiponectin, resistin, and osteocalcin were included in models as logarithmically transformed variables; + indicates adjustments for all covariates in the above model; * indicates adjustments for all covariates in the above model except type 2 DM; β standard regression coefficient; *P* probability value; DM, diabetes mellitus; CVD, cardiovascular disease; BMI, body mass index; HF, hip fracture; 25(OH)D, 25 hydroxy vitamin D; PTH, parathyroid hormone; Ca, calcium; Mg, magnesium; PO₄, phosphate; eGFR, estimated glomerular filtration rate.

TABLE 3: Associations of serum adipokine ratios with serum osteocalcin as dependent variable in linear regression models.

Adjustments	Leptin/Adiponectin ratio		Leptin/Resistin ratio		Adiponectin/Resistin ratio	
	β	<i>P</i>	β	<i>P</i>	β	<i>P</i>
Unadjusted	0.037	0.041	0.055	0.008	0.047	0.098
+ Age, sex, BMI	0.048	0.007	0.079	0.000	0.044	0.114
+ HF type	0.048	0.008	0.079	0.000	0.044	0.115
+ 25(OH)D	0.039	0.028	0.071	0.001	0.045	0.095
+ PTH	0.042	0.019	0.075	0.000	0.048	0.076
+ Ca, Mg, PO ₄	0.041	0.023	0.068	0.002	0.048	0.084
+ eGFR	0.031	0.074	0.057	0.007	0.052	0.042
+ Haemoglobin	0.033	0.064	0.061	0.005	0.052	0.043
+ type 2 DM	0.034	0.055	0.058	0.006	0.035	0.426
+ Hypertension*	0.034	0.060	0.061	0.005	0.053	0.044
+ CVD (any)*	0.034	0.062	0.060	0.005	0.051	0.050
+ CVD (any)	0.034	0.057	0.057	0.009	0.038	0.453

Osteocalcin was included in the models as a logarithmically transformed variable. + indicates adjustments for all covariates in the above model; * Indicates adjustments for all covariates in the above model except type 2 DM; β standard regression coefficient; *P* probability value; DM, diabetes mellitus; CVD, cardiovascular disease; BMI, body mass index; HF, hip fracture; 25(OH)D, 25 hydroxy vitamin D; PTH, parathyroid hormone; Ca, calcium; Mg, magnesium; PO₄, phosphate; eGFR, estimated glomerular filtration rate.

with resistin made this association nonsignificant, indicating that higher resistin levels are incorporated in type 2 DM. Indeed, the patients with type 2 DM have significantly higher serum resistin concentrations (+29.2%) than the rest of the cohort (23.0 ± 11.2 versus 17.8 ± 10.0 ng/mL, $P = 0.010$).

Results of multivariate regression modelling testing the hypothesis that individual adipokine ratios are associated with serum osteocalcin are shown in Table 3. As it would be expected, there was a strong positive correlation between leptin/resistin ratio and serum log-osteocalcin, and it remained significant after all adjustments. There was also

a positive association between leptin/adiponectin ratio and log-osteocalcin before and after adjusting for age, sex, BMI, HF type, and parameters of mineral metabolism, but this association lost significance when further adjusted for eGFR and haemoglobin as well as for comorbidities. In contrast, the adiponectin/resistin ratio was significantly associated with serum log-osteocalcin only in models near fully or fully adjusted.

Taken together, these results indicate that leptin and resistin are independent positive and negative, respectively, determinants of serum OC. Not surprisingly, the

TABLE 4: Independent factors associated with serum adipokine levels in older patients with hip fracture (multivariate linear regression models).

	Leptin		Adiponectin		Resistin	
	β	<i>P</i>	β	<i>P</i>	β	<i>P</i>
Osteocalcin	0.297	0.014	-0.036	0.507	-0.133	0.037
Age	-0.037	0.001	0.015	0.002	0.011	0.050
Sex (m)	-0.598	0.001	-0.184	0.020	0.074	0.437
HF type	0.215	0.163	-0.209	0.002	-0.208	0.010
25(OH)D	-0.006	0.129	0.003	0.155	0.002	0.417
PTH	0.001	0.949	0.020	0.003	0.021	0.006
Ca	-1.056	0.090	-0.216	0.436	0.617	0.059
PO ₄	-0.273	0.057	0.049	0.439	-0.059	0.443
Mg	0.323	0.581	-0.307	0.236	-0.924	0.003
eGFR	-0.009	0.024	0.0011	0.430	-0.001	0.829
Adiponectin	-0.331	0.036	—	—	-0.045	0.621
Leptin	—	—	-0.065	0.036	0.003	0.945
Resistin	-0.063	0.649	-0.018	0.769	—	—
Haemoglobin	0.019	0.036	-0.002	0.429	-0.001	0.767
Type 2 DM	0.232	0.269	-0.120	0.197	0.117	0.261
CVD (any)	0.449	0.004	-0.016	0.827	0.103	0.224

Leptin, adiponectin, resistin and osteocalcin were included in models as logarithmically transformed variables; β standard regression coefficient; *P* probability value; BMI, body mass index; HF, hip fracture; 25(OH)D, 25 hydroxy vitamin D; PTH, parathyroid hormone; Ca, calcium; Mg, magnesium; PO₄, phosphate; eGFR, estimated glomerular filtration rate; DM, diabetes mellitus; CVD, cardiovascular disease.

leptin/resistin ratio significantly and positively correlated with OC predicting 39.2% of the total variance in OC, and the adiponectin/resistin ratio could predict 37.4% of the variance. The partial associations between the leptin/adiponectin, adiponectin/resistin ratios and, OC confirm the independent effects of leptin and resistin on serum OC and show the influence of other factors related mainly to adiponectin.

3.4. Independent Factors Associated with Circulating Adipokines and Their Ratios. We next asked which factors are independent determinants of serum adipokine levels and their ratios. On multiple linear regression analyses and in keeping with previous results, serum OC was a significant predictor of both leptin and resistin, but not adiponectin (Table 4). Age was an independent determinant of each of the three adipokines. None of the adipokines was significantly and independently associated with 25(OH)D. Other parameters independently associated with circulating leptin were male sex, eGFR, adiponectin, (all three inversely), haemoglobin, and CVD (both positively). Serum resistin levels were independently associated with PTH (positively), trochanteric HF type, and serum magnesium (both inversely).

Independent predictors of the three adipokine ratios are shown in Table 5. OC was significantly independently associated with leptin/resistin and adiponectin/resistin ratios but not with the leptin/adiponectin ratio. Age, male sex (both negatively), trochanteric HF type, and haemoglobin (both positively) were the other parameters independently associated with the leptin/resistin ratio; the model explained

44.5% of variance in this ratio. OC was also the only independent predictor of the adiponectin/resistin ratio, but in fully adjusted model it accounted only 5.5% of variance.

4. Discussion

The main findings of this study of 294 consecutive unselected older patients with low-trauma HF are statistically significant correlations between leptin, resistin, and OC indicating complex interactions between adipocytes/monocytes/macrophages and osteoblasts. These have been demonstrated by simultaneous measurements of three major circulating adipokines (leptin, adiponectin, and resistin), their ratios, and serum OC. Multiple linear regression models adjusted for age, gender, BMI, HF type, key factors, or mineral metabolism (calcium, phosphate, magnesium, 25(OH)D, PTH), renal function, and haemoglobin showed that serum OC levels were significantly and positively associated with leptin and negatively with resistin concentrations, but not related to circulating adiponectin levels. Leptin, resistin (or leptin/resistin ratio), age, and eGFR were the only independent predictors of serum OC levels contributing to 39.5% of OC variance. On the other hand, OC was an independent determinant of serum leptin and resistin levels, as well as leptin/resistin and adiponectin/resistin ratios.

Although caution is needed when interpreting results of a cross-sectional study, our data may suggest the presence of adipokine-OC loops, specifically, leptin increases and resistin decreases OC secretion by osteoblasts, whereas circulating OC influences leptin (positive feedback loop) and resistin

TABLE 5: Independent factors associated with serum adipokine ratios in older patients with hip fracture (multivariate linear regression models).

	Leptin/Adiponectin ratio		Leptin/Resistin ratio		Adiponectin/Resistin ratio	
	β	<i>P</i>	β	<i>P</i>	β	<i>P</i>
Osteocalcin	0.511	0.057	0.577	0.009	0.354	0.044
Age	-0.082	0.000	-0.094	0.000	0.010	0.521
Sex (m)	-0.763	0.051	-0.107	0.001	-0.432	0.096
HF type	0.898	0.007	0.782	0.004	-0.028	0.897
25(OH)D	0.024	0.013	-0.010	0.175	0.001	0.910
PTH	-0.082	0.018	-0.047	0.095	-0.020	0.357
Ca	-0.205	0.882	-0.329	0.771	-1.086	0.220
PO ₄	-0.478	0.136	-0.275	0.293	0.121	0.570
Mg	2.077	0.108	2.011	0.058	1.270	0.140
eGFR	-0.015	0.095	-0.011	0.127	0.007	0.208
Haemoglobin	0.026	0.008	0.026	0.001	-0.002	0.728
Type 2 DM	-0.005	0.991	-0.110	0.775	-0.290	0.127
CVD (any)	0.232	0.508	0.141	0.622	-0.169	0.234

Leptin, adiponectin, resistin and osteocalcin were included in models as logarithmically transformed variables; β standard regression coefficient; *P* probability value; BMI, body mass index; HF, hip fracture; 25(OH)D, 25 hydroxy vitamin D; PTH, parathyroid hormone; Ca, calcium; Mg, magnesium; PO₄, phosphate; eGFR, estimated glomerular filtration rate; DM, diabetes mellitus; CVD, cardiovascular disease.

(negative feedback loop) production. These two counterbalancing circuits seem to be important components of a complex homeostatic framework. These results are in line with the current concept that efficient maintenance of metabolic homeostasis depends on interaction between adipose tissue/energy metabolism and skeleton [1–4], but the directions of some associations observed in this as in other clinical studies were opposite to that reported in experimental animals (genetically modified obese rodents).

Although adipokines, especially leptin and adiponectin, two pleiotropic hormones involved in regulation of a large variety of physiological processes, have been extensively studied in recent years, current data on their effects on bone and the adipokine-OC interactions in humans are controversial and the underlying mechanisms not fully understood.

It is now well acknowledged that the effect of leptin on bone is complex and includes different pathways: central inhibition of bone formation through the hypothalamus and brainstem involving β adrenergic, neuromedin U, neuropeptide Y, cocaine, and amphetamine-related transcript and serotonergic systems [35, 46, 47] and direct peripheral (local) enhancement of osteoblastic cell differentiation, proliferation, and bone mineralization [5, 7]. Results from studies of the bone phenotype in animals with leptin deficiency (*ob/ob*) and leptin receptor deficiency (*dbldb*, *fa/fa*) are conflicting. While several groups concluded that leptin acts as a positive regulator of bone formation [48, 49], other suggested a negative (through the hypothalamus) effect on the skeleton [35, 47]. In humans, both positive and negative correlations between leptin and bone mineral density (BMD) [47], as well as with OC [11–21], have been reported.

Our finding that serum leptin is associated with OC is consistent with clinical observations that leptin positively correlated with OC [17] and BMD [15, 18, 50, 51] in different settings, whereas decrease of OC in bone predispose to HF [52]. Our results are also supported by a strong inverse association between serum leptin levels and nontraumatic fracture risk even in normal weight subjects [21], as well as the data that leptin enhances osteoblastogenesis *in vitro* [7, 48, 53], exerts a positive effect in fetal bone formation [54] and reduces bone loss in ovariectomized rats [55].

A positive association between leptin/adiponectin ratio and OC has been reported [17]. In our study, this association disappeared when eGFR, as an independent variable, was included in the regression model, and OC was not an independent predictor of the leptin/adiponectin ratio.

Taken together, it appears that leptin, which is involved in multiple endocrine pathways and exerts a wide spectrum of actions, may lead to opposite effects in different metabolic conditions. The negative effects of leptin on bone may predominant over the positive ones in obesity when leptin resistance occurs or when the serum leptin concentration rises above a certain threshold [50, 56]. In our study group, there were no obese persons and in 1/3 of patients the low serum leptin levels were indicative of malnutrition. In humans, energy deprivation and undernutrition with low leptinaemia are associated with low bone mass [57]. Our data suggests that in underweighted and normal weight persons, lower leptin levels are associated with decrease in OC, which in turn may further decrease leptin production. Leptin may have a therapeutic role in treating osteoporosis in undernourished patients.

Animal studies have indicated that OC regulates insulin metabolism through stimulating the expression of

adiponectin in adipocytes [1–3]. However, the interrelation between OC and adiponectin remains unclear. Adiponectin and its receptors are expressed on osteoblasts, and adiponectin *in vitro* stimulates proliferation and differentiation of osteoblasts [6, 8, 58], and OC enhanced adiponectin expression in cultured adipocytes in a dose-dependent manner [2]. Experimental data *in vivo* (transgenic mice models) demonstrated all three a positive, negative or no effect of adiponectin on bone mass [58, 59]. Clinical studies reported more often a negative association [22, 60, 61], and also a positive [16] or no correlation [62]. Conflicting data on adiponectin-OC relationship [16, 20, 22–30] together with these discrepancies suggest that the effects may differ depending on other metabolic factors and clinical features. In the present study in concordance with other human studies [11, 17, 20, 30], no correlation was observed between serum adiponectin and OC. Serum adiponectin was not associated with BMD of proximal femur in patients with HF [38]. However, we found a positive relationship between adiponectin/resistin ratio and OC, suggesting that a shift in balance towards adiponectin may increase OC production by counteracting the action of resistin. Of note, this correlation was observed only when age, sex, BMI, HF type, parameters of mineral metabolism, and eGFR were included, as independent variables, in the regression analysis model. This indicates that serum adiponectin/resistin ratio is positively associated with OC levels in subjects with similar above-mentioned characteristics (e.g., when the influence of these variables is eliminated).

Our data demonstrate a reciprocal association between resistin and OC which has not been previously described. Resistin is expressed in mature human osteoblasts, and recombinant resistin increases osteoclastogenesis but only weakly affects differentiation of preosteoblasts into osteoblasts [63]. The few clinical studies of resistin-bone relationship provided conflicting data [17, 30, 61, 64]. Our results are in line with observations that resistin inversely correlates with BMD in the hip [64], lumbar spine [30], and radius [65]. Remarkably, the resistin-OC association in our study became significant only after controlling for 25(OH)D and PTH and remained significant after further adjustments for all other covariates except type 2 DM (Table 2), suggesting that this relationship can be masked by parameters of mineral metabolism. Indeed, our data showed that PTH and magnesium were independent determinants of circulating resistin but not leptin (Table 5) providing a potential explanation for the “masked” effect.

Of note, after adjusting for type 2 DM, the association between OC and resistin became nonsignificant, suggesting that common signalling and metabolic pathways for OC and resistin contribute to DM. In line with other studies [66–69], we found that serum resistin levels in patients with type 2 DM were significantly higher compared to the rest of the cohort. These observations are consistent with growing evidence that resistin may be a potential mediator of DM [70–73] acting, at least partially, through OC.

The relationships between adipokine ratios and OC have not been systematically examined previously. In this study, after full adjustment, a significant interrelation

between the leptin/resistin ratio and OC and between adiponectin/resistin ratio and OC was found. However, analyses of these ratios did not yield additional information compared to either leptin or resistin measurements.

It should be pointed out that multiple regression analysis explained only 39.5% of the variance in serum OC, indicating that factors other than leptin and resistin significantly influenced the level of OC. We found that both eGFR and age are independently associated with serum OC as well as leptin levels, and age is an independent determinant of resistinaemia. Consistent with other studies, ours showed that deterioration of kidney function was associated with higher OC [22] and leptin [74] levels. There is an age-dependent decrease in proliferation and differentiation of human osteoblasts, and the highest OC levels have been reported during adolescence [75]. However, in adults, no correlation between OC and age was found in some studies [11], while other described decreased OC levels with age [76]. In about half (53.3%) of our patients, the serum OC concentration was low (lower than the low limit of the reference range) but it was significantly and positively associated with age. It is reasonable to consider that renal dysfunction may at least partially explain this association, as we observed a strong negative correlation between age and eGFR ($r = -0.313$, $P = 0.001$) and eGFR was markedly decreased (<60 mL/min./1.73 m²) in 42.6% of our patients.

The complex interplay of many metabolic, renal, and age-related factors may account for some of the discrepancies in the literature in regard to adipokine-OC interactions. It is possible that various combinations of these factors are causing distinct positive or negative effects. Further complicating the matter, adiponectin and resistin (as well as leptin) have been shown to be neuroendocrine hormones acting directly on the brain [77–81], but in contrast to leptin, the centrally mediated effects of adiponectin and resistin on osteoblast functions are unknown. Moreover, receptors for resistin and OC still remain unidentified. To develop an integrated understanding of adipokine-bone interaction, a lot more work is needed to be done. There is a growing body of evidence demonstrating that in osteoporosis impaired bone metabolism, including OC production and secretion, does not exist in isolation. It reflects the alterations in a highly complex homeostatic system. Our data indicate the existence of bidirectional leptin-OC (positive) and resistin-OC (negative) relationships as a part of a complex energy metabolism-bone network in older patients with HF. Figure 1 represents the complex interactions of OC with adipokines depicting independent significant associations between OC, circulating adipokines, their ratios, age, and renal status in older patients with HF. Further examination of the role of these interactions in osteoporotic fractures and metabolic disorders was warranted.

In regard to the differences in OC-adipokine interactions observed in humans and rodents, it should be noted that, firstly, the *Esp* gene, specifically studied in knockout mouse models, is a pseudogene in humans [82]. No functional *Esp* gene has been identified in humans, although a close homologue of *Esp* is expressed in human osteoblasts [83]. Secondly, in genetically modified rodents, changes in OC

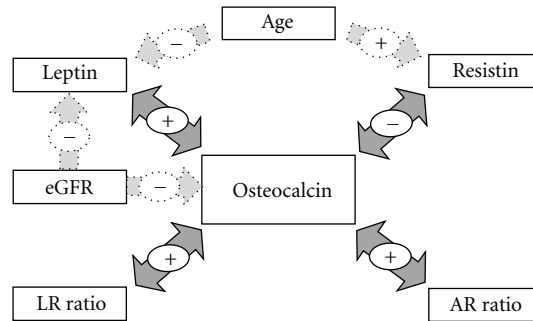


FIGURE 1: Schematic representation of independent significant associations between serum osteocalcin, adipokines, their ratios, renal status, and age in older patient with hip fracture. LR, leptin/resistin ratio; AR, adiponectin/resistin ratio, eGFR, estimated glomerular filtration rate. Bidirectional interactions exist between leptin and osteocalcin (positive), between resistin and osteocalcin (negative) and as a consequence between leptin/resistin ratio and osteocalcin and between adiponectin/resistin ratio and osteocalcin (both positive). Age is an independent determinant of circulating levels of resistin (positive association) and leptin (negative associations), whereas renal function (eGFR) is inversely associated with osteocalcin and leptin.

and adipokine levels are much larger than in clinical observations. Thirdly, the compensatory mechanisms caused by genetic manipulations are not presented in humans. Fourthly, the effects of ageing and comorbidities have not been addressed in animal studies.

The notable strength of this study is simultaneous assessment of three circulating adipokines and OC in the same cohort and adjustment for a wide range of confounding factors, the major limitations of previous studies. However, multiple comparisons in multivariate regression analysis may potentiate multicollinearity. After Bonferroni and Sidak adjustments, all determinants preserved statistical significance, and in all our models (Tables 2–5), the variance inflation factor was between 1.21 and 1.27 indicating that the amount of multicollinearity was not significant. The main limitation of our study is its cross-sectional design which precludes conclusions regarding causality. Another potential limitation of this study is that only total OC and total adiponectin have been measured. The animal and *in vitro* studies showed that uncarboxylated OC exerts an effect on glucose homeostasis and energy metabolism [1]. However, other studies reported that both carboxylated and uncarboxylated forms of OC and total OC are associated with glucose metabolism and insulin resistance [11, 23, 24, 76]. Similarly, some studies concluded that high-molecular weight (HMW) adiponectin is a better predictor of insulin resistance and metabolic syndrome, while other studies did not find a significant difference between HMW and total adiponectin in this regard. We cannot exclude the possibility that measurements of specific forms of OC and adiponectin may provide different results. Finally, our study population represents almost exclusively elderly Caucasians, and the results may have limited applicability to other age and ethnic groups.

In conclusion, in older patients with HF, leptin is directly and resistin inversely associated with circulating OC, and OC is a significant independent determinant of both serum leptin (positive) and resistin (negative) concentrations. These suggest bidirectional interactions (crosstalk) between leptin, resistin and OC as a part of a complex homeostatic

system regulating bone and energy metabolism. Our data do not support an independent link between adiponectin and OC in these patients. Further studies should be performed to evaluate the role of leptin-OC and resistin-OC axes in osteoporotic fractures and comorbid conditions such as cardio- and cerebrovascular diseases, diabetes, dementia, malnutrition, all of which are common in the elderly and have been shown to be associated with alterations in serum adipokine and OC levels.

Conflict of Interests

The authors declare that there is no conflict of interests.

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