Universidade Federal do Rio Grande do Sul

Faculdade de Medicina

Programa de Pós-Graduação em Ciências Médicas: Endocrinologia

Níveis séricos e expressão gênica de leptina, adiponectina e aromatase em tecido adiposo de mulheres com a síndrome dos ovários policísticos

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Tese apresentada ao Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, como requisito parcial para obtenção do título de Doutor

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Porto Alegre, dezembro de 2010.

Agradecimentos

A minha orientadora, Profa. Dra. Poli Mara Spritzer, agradeço pelo seu exemplo de sucesso, pela confiança e responsabilidade em mim creditadas. Obrigada por ter me contagiado com o seu entusiasmo pela vida acadêmica e científica.

A minha co-orientadora, Profa. Dra. Débora Martinho Morsch, pela amizade, atenção e carinho.

À Miriam Sant'Helena por sua amizade, dedicação e carinho.

À Universidade Federal do Rio Grande do Sul e ao Hospital de Clínicas de Porto Alegre por me proporcionarem os meios para uma formação de qualidade.

Aos meus colegas da Unidade de Endocrinologia Ginecológica do Serviço de Endocrinologia do Hospital de Clínicas de Porto Alegre: Andrea Prestes Nácul, Betânia Rodrigues dos Santos, Bruna Cherubini Alves, Luiz César Fernandes Vilodre, Denusa Wiltgen, Fabian Jonas Nickel, Fabíola Satler, Fabrício Mattei, Gislaine Krollow Casanova, Kristhiane Di Domenico Cunha, Maria Augusta Maturana, Maria Cristina Gomes Matos, Mariana Kirjner Toscani, Natália Altmeyer Goulart, Polyana Sartori Maier, Ramon Bossardi Ramos, Raquel Amaral Vieira, Roberta Fernandes Franz, Roberta Martins Costa Moreira, Scheila Karen Graff, Simone Matiello, Suzana Ruschel, Tassia Maciel, Thais Rassia, Thais Wallau e Vânia Teixeira Andrade. Obrigada pela amizade e por formarmos uma equipe de trabalho responsável e descontraída.

Aos colegas do Laboratório de Biologia Molecular do Serviço de Endocrinologia do Hospital de Clínicas de Porto Alegre pela agradável convivência.

Ao Dr. Carlos Franco Voegeli e equipe do Laboratório Central de Análises Clínicas do Complexo Hospitalar Santa Casa de Porto Alegre pela compreensão ao dividir minha dedicação entre a atividade profissional e a formação acadêmica.

Ao Prof. Dr. Francisco Lhullier pelo constante assessoramento profissional.

À Dra. Adriana Reis do Nascimento pela dedicação e orientação na busca do meu autoconhecimento.

À Simone Radavelli-Bagatini pelo seu exemplo de superação e atenciosa amizade.

Às amigas Carine Ghem, Estefânia Perin Fonseca, Marta Regina Goeltzer, Marialva Gonçalves e Patrícia Tenconi pelo amor e incentivo singulares.

A minha família, alegria da minha vida:

Ao meu amor, Cristiano de Oliveira Severo, agradeço pelo seu carinho e incentivo. Obrigada pela compreensão e paciência com esta jornada.

A minha irmã querida, Patrícia Bünecker Lecke, pelo amor e estímulo. Obrigada por dividir comigo a sua corajosa bagagem de vida!

Aos meus pais, Erci Bünecker Lecke e Christiano Walter Lecke, pelo exemplo. Obrigada por compartilharem comigo os momentos mais importantes. Agradeço pela amizade, amor, compreensão, conforto e dedicação incondicionais!

Esta Tese de Doutorado segue o formato proposto pelo Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, Metabolismo e Nutrição, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, sendo apresentada na forma de três manuscritos sobre o tema da Tese:

- Artigo de revisão: "Leptin and adiponectin in the female life course" (submetido à Brazilian Journal of Medical and Biological Research, 2010)

- Artigo original 1: "Abdominal subcutaneous fat gene expression and circulating levels of leptin and adiponectin in polycystic ovary syndrome" (submetido à Fertilty Sterility, 2010)

- Artigo original 2: "Association between blood pressure and CYP19 gene expression in subcutaneous adipose tissue of women with polycystic ovary syndrome"

SUMÁRIO

RESUMO

A síndrome dos ovários policísticos (polycystic ovary syndrome – PCOS) constitui o distúrbio endócrino mais comum em mulheres em idade reprodutiva, afetando cerca de 5 a 10% das mulheres em todo o mundo. Caracteriza-se por hiperandrogenismo e disfunção ovariana, incluindo oligo-anovulação e/ou ovários policísticos na ausência de outras doenças que causem hiperandrogenismo. Também importante é o reconhecimento da PCOS como uma disfunção metabólica, manifestada por obesidade abdominal, resistência à insulina, dislipidemia e hipertensão. Estes fatores aumentam o risco para diabetes tipo 2 e, provavelmente, doença cardiovascular. O tecido adiposo secreta adipocinas e pode mediar inúmeros processos fisiológicos. Entre as adipocinas, a leptina e a adiponectina têm sido associadas com índice de massa corporal (IMC), ação da insulina e metabolismo da glicose. Além disso, o tecido adiposo armazena, mobiliza e forma esteróides a partir de interconversões de vários metabólitos hormonais. A aromatase é a enzima-chave para a síntese de estrogênios a partir de androgênios e está presente no tecido adiposo. Evidências indicam associações entre hormônios sexuais e hipertensão arterial sistêmica. Nas mulheres com PCOS, a hipertensão tem sido relacionada ao excesso de androgênios e à resistência à insulina.

No presente estudo, avaliamos os níveis séricos de leptina e adiponectina, calculamos a relação leptina/adiponectina (L/A), bem como determinamos a expressão gênica de leptina, adiponectina e aromatase no tecido adiposo subcutâneo de mulheres com PCOS e de mulheres controles não hirsutas, com ciclos ovulatórios e pareadas para IMC. Encontramos níveis mais elevados de leptina sérica nas pacientes PCOS com sobrepeso/obesidade em relação com mulheres de peso normal, enquanto que as concentrações de adiponectina foram semelhantes em todos os subgrupos. A relação L/A foi maior nos subgrupos sobrepeso/obesidade quando comparados com o grupo controle de peso normal. A expressão gênica de leptina no tecido adiposo subcutâneo foi maior nas mulheres PCOS com sobrepeso/obesidade em relação às controles de peso normal, enquanto que a expressão gênica de adiponectina foi semelhantes entre os grupos. Na análise de regressão múltipla, o percentual de gordura corporal contribuiu significativamente para a relação L/A em PCOS, independentemente do IMC e do índice de androgênios livres. O RNAm da aromatase no tecido adiposo foi significativamente mais elevado no grupo de PCOS hipertensas (NCEP/ATP III 2001)

quando comparado com as PCOS normotensas e com o grupo controle. Uma correlação positiva entre a expressão gênica de aromatase e a pressão arterial sistólica e diastólica foi observada nas pacientes com PCOS.

Em conclusão, os dados do presente estudo sugerem que as alterações observadas na secreção de adipocinas parecem estar mais relacionadas à adiposidade do que ao excesso de androgênios na PCOS. Além disso, o excesso de androgênios e a hiperinsulinemia podem ter algum papel nos mecanismos moleculares que ativam a transcrição do RNAm da aromatase no tecido adiposo de mulheres com PCOS.

ABSTRACT

Polycystic ovary syndrome (PCOS), the most prevalent endocrine disorder in women of reproductive age, affects 5 to 10% of women worldwide. It is characterized by hyperandrogenism and ovarian dysfunction, including oligo-anovulation and/or polycystic ovaries in the absence of other diseases affecting pituitary and/or adrenal glands. Also important is the recognition of PCOS as a metabolic disorder, manifested by abdominal obesity, insulin resistance, dyslipidemia and hypertension. These factors increase the risk for type 2 diabetes and probably for cardiovascular disease. Adipose tissue has been recognized as an active endocrine organ and a secretory mediator of numerous physiological processes. The adipokines leptin and adiponectin have been associated with body mass index (BMI), insulin action, and glucose metabolism. In addition, adipose tissue is a reservoir of steroids, a center of sexual hormone conversion and a source of estrogen. Aromatase is the key enzyme for estrogen synthesis from androgens and is present in adipose tissue. Evidences indicate there are associations between sex hormones and arterial hypertension. In women with PCOS, hypertension has been linked to androgen excess and insulin resistance.

In the present study, we assessed leptin and adiponectin serum levels and the leptin to adiponectin (L/A) ratio and evaluated the gene expression of leptin, adiponectin and aromatase in subcutaneous adipose tissue from PCOS and BMI-matched non-hirsute, ovulatory control women. We found higher leptin serum levels in overweight/obese PCOS patients than in normoweight women, while adiponectin concentrations were similar in all subgroups. L/A ratio was higher in overweight/obese subgroups than in normoweight controls. Subcutaneous leptin gene expression was higher in overweight/obese PCOS women than in normoweight controls, while the adiponectin gene expression was similar in all groups. On multiple regression analysis, percentage of body fat contributed significantly to L/A ratio in PCOS, independently of BMI and free androgen index. Subcutaneous aromatase mRNA was significantly higher in the hypertensive (NCEP/ATP III 2001) PCOS than in normotensive PCOS and control groups. A positive correlation between aromatase gene expression in subcutaneous fat with systolic and diastolic blood pressure was observed in PCOS patients.

In conclusion, results from our study suggest that altered adipocyte secretion seems to relate to adiposity rather than to androgen excess in PCOS. In addition, our

data suggest that androgen excess and hyperinsulinemia may play a role on the molecular mechanisms that activate aromatase mRNA transcription in abdominal fat tissue in women with PCOS.

Parte I

Artigo de revisão:

Leptin and adiponectin in the female life course

Leptin and adiponectin in the female life course

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Conflicts of interest: none to declare

Financial support: This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq INCT 573747/2008-3), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES 1289/05) and Fundo de Apoio à Pesquisa do Hospital de Clínicas de Porto Alegre (FIPE-HCPA 340/2004), Brazil.

Abstract

The adipose tissue secretes a variety of adipokines, including leptin and adiponectin, which are involved in endocrine processes regulating glucose and fatty metabolism, energy expenditure, inflammatory response, immunity, cardiovascular function, and reproduction. The present article describes the fluctuations in circulating leptin and adiponectin as well as their patterns of secretion in women from birth to menopause. During pregnancy, leptin and adiponectin seem to act in an autocrine/paracrine fashion in the placenta and adipose tissue, playing a role in the maternal-fetal interface and contributing to glucose metabolism and fetal development. In newborns, adiponectin levels are two to three times higher than in adults. Full-term newborns have significantly higher leptin and adiponectin levels than preterms, while small for gestational age babies have lower levels of these adipokines than adequate for gestational age newborns. However, with weight gain, leptin concentrations increase significantly. Children between five and eight years of age experience an increase in leptin and decrease in adiponectin regardless of body mass index, with a reversal of the newborn pattern for adiponectin: plasma adiponectin levels at age five are inversely correlated with percentage of body fat. In puberty, leptin plays a role in the regulation of menstrual cycles. In adults, it has been suggested that obese individuals present both leptin resistance and decreased serum adiponectin levels. In conclusion, a progressive increase in adiposity throughout life seems to influence the relationship between leptin and adiponectin in women.

Introduction

The adipose tissue is currently recognized as a dynamic organ because of its capacity to secrete hormones and cytokines. White adipose tissue, the predominant form found in adults (while brown fat is mainly found in neonates), consists of preadipocytes and adipocytes together with macrophages, endothelial cells, fibroblasts and leukocytes (1), and secretes a variety of adipokines. These adipokines are involved in a range of endocrine processes that regulate glucose and fatty metabolism, energy expenditure, inflammatory response, immunity, cardiovascular function and reproduction, among others (2-6).

Several aspects relating to leptin and adiponectin, including metabolic, clinical, and pharmacological characteristics, have been extensively studied. However, the behavior of these adipokines in the course of life has deserved less attention. In the present article the fluctuations in circulating leptin and adiponectin as well as their patterns of secretion are described from birth to menopause in order to trace a physiological profile of these adipokines in the female life course.

Leptin and Adiponectin

Since its discovery in 1994, leptin has opened a wide field for research on the biology of adipocytes, including metabolic and endocrine roles as well as the relationships between adipose secretagogues and peripheral tissues. Leptin, the *ob* gene product encoded by chromosome 7q31.3, provides a molecular basis for the lipostatic theory of the regulation of energy balance (7). It has become clear that leptin also plays an important role in reproduction, both during pregnancy and early neonatal life (7). Leptin is produced by adipocytes and also by the placenta (8). It has been shown to act as an afferent satiety signal regulating appetite and weight in humans and rodents (9, 10). Leptin circulates as a monomeric 16 kDa protein or bound to leptin binding proteins, forming large high molecular weight complexes (11), and acts through the leptin receptor (Ob-R). The Ob-R family is a large single membrane spanning protein belonging to the class I cytokine receptor family (12, 13). The Ob-R gene encodes at least five alternatively spliced variants. The long form of Ob-R is considered the active leptin receptor, while the shorter forms lack the membrane spanning region and are supposedly involved in leptin transport (14) (Figure 1). Ob-R is found in regions of the hypothalamus, gonadotropic cells of the anterior pituitary gland, ovarian interstitial cells, theca and granulosa cells of ovarian follicles, endometrium and the Leydig cells

from the seminiferous tubules (15). Moreover, Ob-R has been identified in the kidneys, heart, lungs, liver, and skeletal muscle (16).

Adiponectin was first discovered in 1995 as a protein released exclusively by adipose tissue (17). Because it was simultaneously found by several laboratories, adiponectin received various names – adipocyte complement-related protein of 30 kDa (Acrp30), adipose-specific gene adipoQ (AdipoQ), adipose most abundant gene transcript 1 (apM1) and gelatin-binding protein of 28 kDa (GBP28) (18). Adiponectin is the protein product of the APM1 gene transcript, which is located at chromosome 3q27, close to the locus for type 2 diabetes and adiposity (19, 20). This adipokine is secreted by adipose tissue as a trimer of low molecular weight (LMW), a combination of two trimers of middle molecular weight, or as six trimers of high molecular weight (HMW) and circulates either as a trimer or an oligomer (Figure 2). HMW forms are the predominant and active form in human serum (21, 22). Adiponectin increases fatty acid oxidation in adipose tissue, liver and muscle, enhancing insulin sensitivity, and inhibits inflammatory mediators and the expression of adhesion molecules within the vascular wall, lowering atherogenic risk (1, 21, 23, 24). Adiponectin receptors have been identified predominantly in muscle (AdipoR1) and liver cells (AdipoR2), although in humans they are expressed ubiquitously in the body and brain (6, 18, 22, 25, 26).

In most studies, leptin and adiponectin circulating levels have been determined by enzyme-linked immunosorbent assays or enzyme immunoassay or, alternatively, radioimmunoassay associated or not with *in house* techniques. While many studies report assay sensitivity and intra and interassay coefficients of variation, quality control measurements are rarely described.

Pregnancy and Intrauterine and Neonatal Development

During pregnancy, development of the neonate is influenced by critical interactions among genes, epigenetic changes, hormones, nutrition, intrauterine environment, and early pre- and postnatal events.

Previous studies have reported that the increased leptin levels in pregnant women result from placental secretion, in addition to adipose tissue production (8, 27, 28). The higher leptin levels during pregnancy have also been attributed to weight gain and to an increase in body mass index (BMI), as well as changes in hormone levels that might stimulate leptin secretion (e.g. insulin, estrogens and human chorionic gonadotropin) (29). Trophoblastic cells, the predominant placental cell type, are the

most likely source of placental leptin (8, 30, 31). Caminos et al. (32) were the first to demonstrate the presence of adiponectin and AdipoR2 in human and rat cytotrophoblast and syncytiotrophoblast placental cells. Chen et al. (1) described the production of adiponectin and the presence of both adiponectin receptors in the human placenta, indicating that the placenta was also able to secrete adiponectin *in vitro*. It appears that leptin and adiponectin act in an autocrine/paracrine fashion in the placenta, playing an important role in the maternal-fetal interface and contributing to glucose metabolism and fetal development during pregnancy (1, 26, 29, 33).

In turn, if prematurity or fetal growth restrictions prevent the fetus from acquiring sufficient adipose tissue, changes are observed in the cord blood adipokine profile. Martos-Moreno et al. (34) have reported that full-term newborns had significantly higher leptin and adiponectin levels than preterms, while babies who were small for gestational age had lower levels of these adipokines than adequate for gestational age newborns. In that study, gestational age was the main predictor of adiponectin levels, while birth weight was independently correlated with leptin cord blood levels. Nevertheless, increases in fetal weight also enhanced cord blood adiponectin levels, indicating that the negative influence of fat mass on adiponectin secretion is absent in the fetus (34).

Mantzoros et al. (35) confirmed that adiponectin levels are two to three times higher in neonates than in adults. One possible explanation is that neonates have significantly less fat mass than children and adults, and that their fat mass consists mainly of subcutaneous fat and brown adipose tissue, which is atrophic in adults. In this regard, other investigators have reported a positive association between cord blood total adiponectin as well as HMW adiponectin levels (36) with newborn birth weight, BMI, birth weight/length ratio, and leptin levels (37-40), even though this was not confirmed by others (41). Some studies also provide evidence that leptin may be found in high concentration in cord sera of full term newborn infants, being closely related to birth weight and fat accumulation (42-45). These findings imply that the relationship between leptin and body fat mass may develop early in life.

Previous studies on preterm infants have demonstrated that serum leptin concentrations were significantly lower compared with appropriate for gestational age neonates (46, 47). However, as weight gain progressed, leptin concentrations increased significantly (8, 29, 48). It has been speculated that these increased leptin levels may derive from the increase in adipose fat typically observed after the under-nutrition

period in the preterm, suggesting a possible involvement of leptin in early development of insulin resistance (49-51). A series of epidemiological human and experimental animal studies have shown that exposure to inadequate (high or low) nutrition levels during fetal and postnatal life, as well as reduced fetal growth followed by excessive postnatal catch-up in height and particularly in weight, increase the risk of obesity, insulin resistance and type 2 diabetes in child and adult life (52, 53). The decrease in adiponectin in small-for-gestational-age infants with the greatest weight gain catch-up was also reported as a risk for subsequent development of visceral fat and insulin resistance (26, 40).

Perturbations in fetal nutrient supply affect adipocyte levels. Muhlhausler et al. (52) have proposed that prenatal over-nutrition promotes the growth of the subcutaneous fat depot, leading to an increase in leptin secretion and serum levels. In fact, hyperleptinaemia during neonatal life may program leptin resistance, with long-term adverse consequences of the hypothalamic circuits regulating energy balance via dual effects on food intake and energy expenditure (6, 54). Some studies have focused on newborns from obese mothers, demonstrating an increased risk for obesity and insulin resistance, as well higher levels of leptin and markers of inflammation than in newborns from lean mothers (55).

A study with 100 mother/newborn pairs has tried to verify the production of leptin in the uterus and its role in neonatal development. Although maternal serum leptin concentrations correlated with measures of pre and post pregnancy adiposity, there was no association between serum leptin levels in the newborn (8), an observation confirmed by others (13, 42). Interestingly, Hassink et al. (8) demonstrated that 13% of newborns had higher serum leptin concentrations than their mothers, suggesting that the fetus may produce its own leptin with associated leptin resistance and/or the placenta is the major source of leptin production for the fetus. However, Ho et al. (46) have shown a correlation between serum leptin concentrations in neonates with maternal weight and BMI within 24 hours from birth, suggesting that maternal and even placental production of leptin may be involved in the early regulation of fetal metabolism and growth. As in previous studies, no association was demonstrated between neonatal adiponectin levels and maternal age and pre-pregnancy BMI, suggesting that neonatal adiponectin levels are largely independent of maternal influence (37). Bozzola et al. (49) evaluated both total and isoform adiponectin circulating levels in a cohort of infants, showing a significant increase from birth up to the first month of life, and a decrease at one year of

age in all subjects. The investigators argue that the observed fall in serum adiponectin levels may be a consequence of increased adiposity, confirming the negative association between serum adiponectin levels and percentage of body fat, as is the case in older children (49, 56).

Childhood and Puberty

In context of the worldwide epidemics, the role of adipokines to predict obesity in childhood and puberty may become biologically and potentially important for public health.

Between five and eight years of age, metabolic health seems to improve regardless of BMI, with an increase in leptin and decrease in adiponectin (57). In fact, adiponectin levels in childhood show a marked reversal of the positive body mass correlations observed during the newborn period: plasma adiponectin levels at age five are inversely correlated with percentage of body fat. This inverse association between BMI and adiponectin becomes even more marked in older children (40, 58). Previous studies in children of different ethnicities have reported that low plasma adiponectin concentrations were closely related to hyperinsulinemia (58-61), leading to a long-term increased risk for diabetes.

There is a developmental increase in adipose tissue leptin expression and secretion during childhood, reaching maximal capacity in puberty/adulthood. The temporal correlation between increases in leptin concentration and the initiation of LH pulsatility over peripuberty has been studied in several species. It is well recognized that leptin stimulates the gonadotropin releasing hormone (GnRH) pulse generator and in turn LH pulses. This adipokine is also secreted in a pulsatile manner in ovulatory women: a peak at night followed by a gradual change in LH pulsatility until reaching a nadir in the morning (11, 62).

Fenichel et al. (62) performed a 24 hour study of leptin, LH and metabolic rate in a group of young to middle-age ovulatory women, showing that the nightly leptin peak was associated with a decrease in LH pulse frequency and an increase in LH amplitude, and that the acute changes in LH pulses were accompanied by a fall in metabolic rate. These results suggest that in normal individuals a single metabolic cycle regulates the leptin peak and the slowing of LH and metabolic rate. Therefore, leptin may be regarded as a link between modulation of food intake and energy expenditure and the reproductive system. Leptin may signal to the body that energy reserves are

adequate for reproduction, representing one of the primary mechanisms linked to the onset of puberty (15, 16, 63). In fact, a minimal leptin concentration is necessary to activate the hypothalamus–pituitary–gonadal axis, triggering puberty and maintaining reproductive function. However, hyperleptinaemia, such as occurs in obesity, might impair gonadal steroidogenesis and reproduction (29). The role of leptin in the regulation of menstrual cycles is further supported by studies demonstrating that menstrual irregularities and amenorrhea are common in women with very high or very low leptin concentrations (15, 29). However, there is evidence that leptin functions as a permissive, and not as a definitive factor in the onset of puberty, working in concert with other metabolic signals (29).

Adulthood and Post Menopause

In adults, circulating levels of leptin increase in the presence of total and central obesity and metabolic disturbances (16, 64-66), while adiponectin is inversely associated with the degree of adiposity. It is suggested that obese individuals present both leptin resistance, which antagonizes the metabolic action of insulin, and decreased serum adiponectin levels, increasing the risk for type 2 diabetes, coronary artery disease and metabolic syndrome (67-69).

Leptin concentrations may vary during the physiological menstrual cycle, exhibiting a monthly rhythm in ovulatory women. Previous studies with middle-aged, normoweight ovulatory women demonstrated that serum leptin levels were significantly lower during the follicular phase of the menstrual cycle as compared to the midcycle and luteal phases (70). Hardie et al. (71) have also demonstrated higher serum leptin concentrations during the luteal phase. However, as observed by our group, young adult women show great variability in leptin levels that may mask the subtle differences observed in phases of the menstrual cycles (Figure 3). Serum leptin levels also vary in accordance with changes in serum estradiol concentration in these young women, despite the absence of variation in total body fat and weight (72). In the same way, food intake also exhibits a similar monthly rhythm in ovulatory women, as documented in the premenstrual dysphoric syndrome: a nadir around the time of ovulation when estrogen is elevated, and an increase following ovulation and during the luteal phase when progesterone is dominant (73, 74).

Asimakopoulos et al. (70) showed no association between serum adiponectin levels, sex steroids, and others adipokines in middle-age normoweight ovulatory

women, demonstrating that the mean adiponectin concentrations remained practically stable during the menstrual cycle. In addition, no significant changes in serum adiponectin concentrations were reported by Kleiblova et al. (75) in relation to sex hormones or to the degree of insulin sensitivity during the menstrual cycle. In this regard, Rouen et al. (76) observed no variation in adiponectin secretion along the menstrual cycle. These findings suggest that adiponectin is probably not related to reproductive functions in adult women. However, Galvan et al. (77) reported that serum adiponectin concentration was lower in the post ovulatory phase in healthy ovulatory women.

It is believed that numerous factors, including body weight and fat composition, insulin resistance and associated metabolic disturbances, as well as diet and physical activity are related to serum leptin and adiponectin concentrations in different manners (15, 16, 64, 67, 68). The way leptin and adiponectin regulate metabolic homeostasis is different in some aspects and complementary in others (78).

Evidence suggests that the risk for insulin resistance increases after menopause and may be related to the decline in estrogen (77). In fact, postmenopausal accumulation of central abdominal fat secondary to estrogen suppression contributes to insulin resistance which in turn influences serum adipokine concentrations (79).

Previous studies have shown contradictory results regarding serum adiponectin levels after menopause: some observed a decrease (79, 80), while others showed unchanged (81-83) or even increased serum adiponectin concentrations (84). In a cross sectional study with amenorrheic Mexican women presenting climacteric symptoms and not using hormone therapy, serum adiponectin levels showed no significant variation when compared with normal menstrual cycle women (85). In addition, serum adiponectin levels were significantly decreased while serum leptin levels were increased in obese postmenopausal women compared to normoweight women (86). Adiponectin concentration appears to be higher in normoweight than in overweight recent postmenopausal women (76). Taken together, these data support the idea of an inhibitory role for both estrogen and body fat composition on this adipokine secretion.

In conclusion, a progressive increase in adiposity throughout life seems to influence the relationship between leptin and adiponectine in women (Table 1, Figure 4). Further studies are needed to elucidate the relevance of leptin and adiponectin to fetal/neonatal development, as well as possible interactions of these adipokines with fetal/neonatal growth and energy homeostasis. Also, while the role of leptin in puberty

onset is well established, possible interactions between adiponectin and reproductive onset remain to be elucidated.

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Table 1: Serum leptin and adiponectin concentrations during the female life course

Phase of life	Leptin	Adiponectin
Neonate		
Small for gestational age and/or under-nutrition	↓	↓
Adequate for gestational age	↔	↔
Large for gestational age and/or over-nutrition	↑↑	↑↑
Childhood	↔	↔
Puberty	↑	↔
Adulthood		
Lean women	↔	↔
Overweight/obese women	↑	↓
Post menopause		
Lean women	↔	↑↓
Overweight/obese women	↑	↓

(↑) Increased; (↓) Decreased; (↔) No alteration; (↑↓) Not established.

Figure 1:

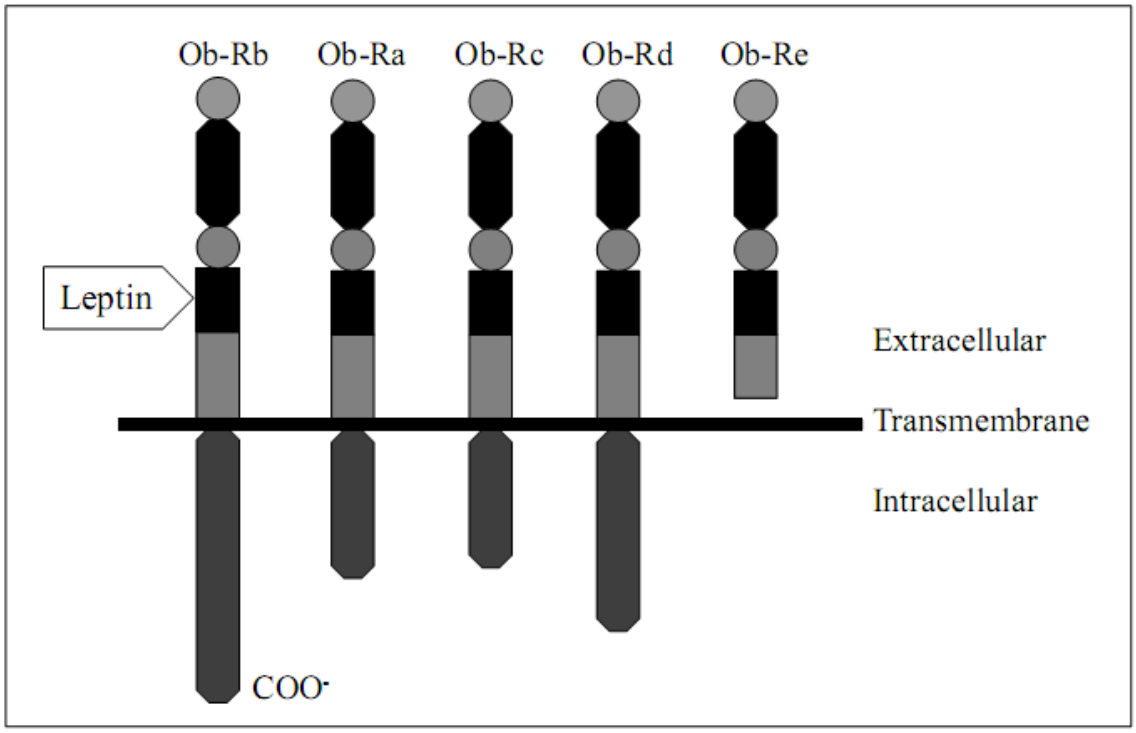

Ob-Rb
Ob-Ra
Ob-Rc
Ob-Rd
Ob-Re
Leptin
Extracellular
Transmembrane
Intracellular
COO-

Figure 2:

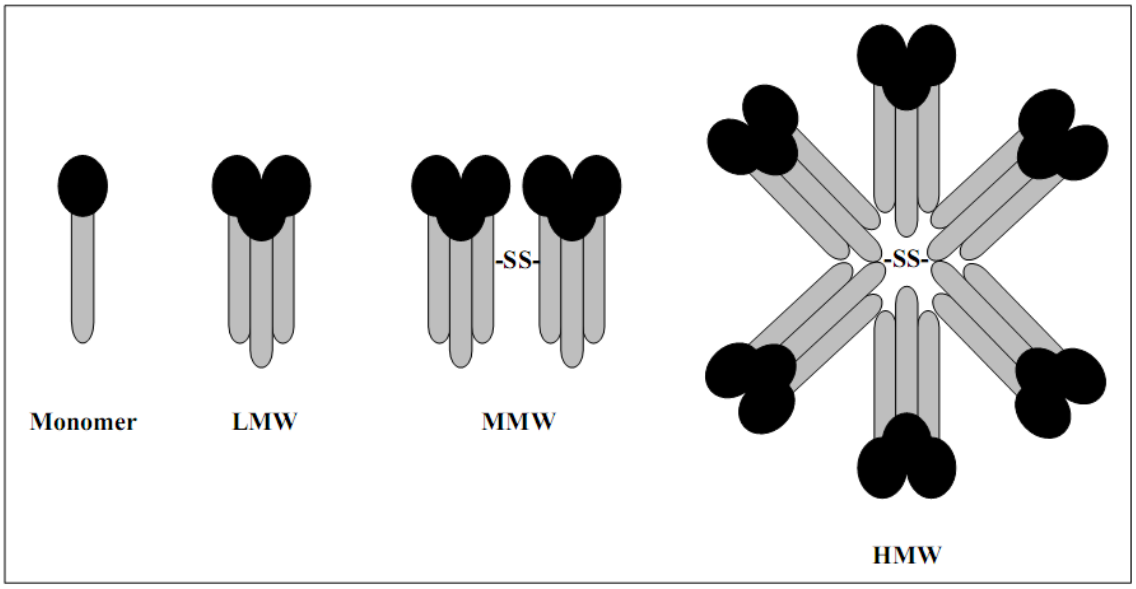
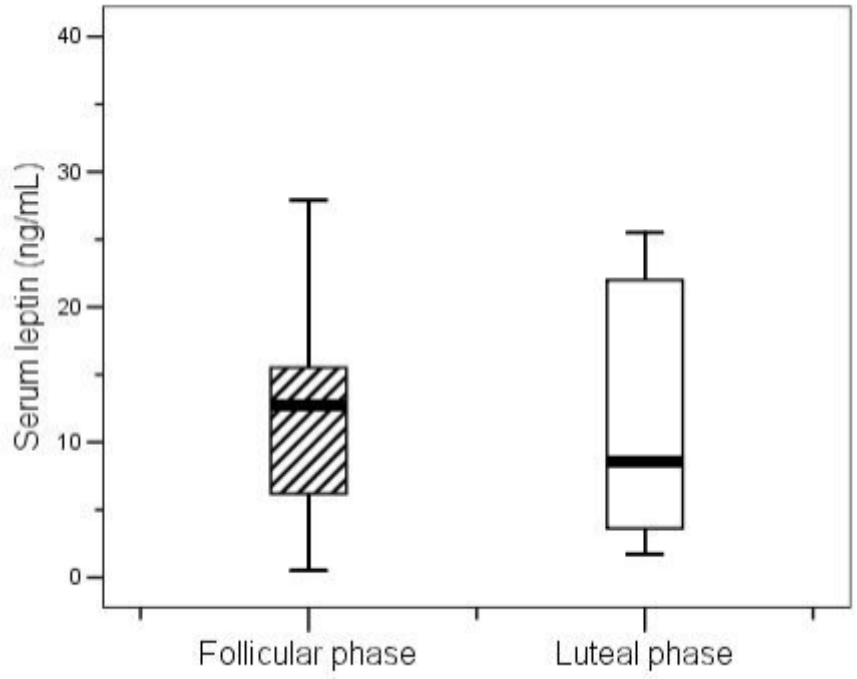


Figure 3:



p = 0.436 by Mann-Whitney *U* test.

n = 28.

Figure 4:

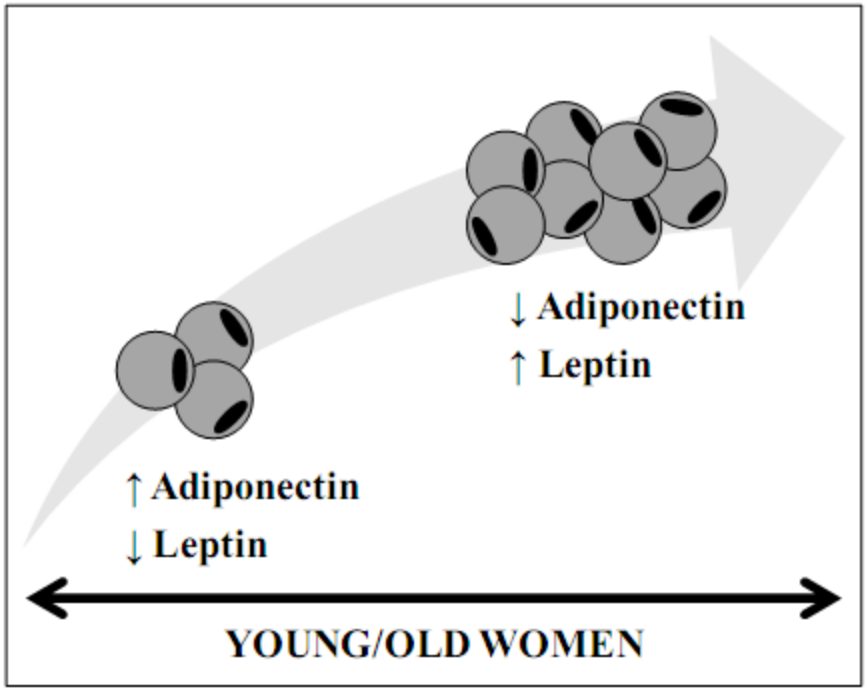


Figure legends

Figure 1: Representation of leptin receptor forms (Ob-R). All share identical extracellular ligand-bind domains, differing at the C-terminus. The long form of the Ob-R (Ob-Rb) is considered the active leptin receptor, activating intracellular signal transduction pathway.

Figure 2: Representation of the circulating adiponectin forms. LMW = low molecular weight; MMW = middle molecular weight; HMW = high molecular weight; SS = disulfide bond.

Figure 3: Leptin levels in normal weight, young adult cycling women in the follicular or luteal phase. Values are expressed as median and interquartile range (25% to 75%).

Figure 4: Leptin and adiponectin serum levels in different phases of the female life course. Adiponectin serum concentration decrease, while leptin serum levels markedly increase with adiposity.

Parte II

Artigo original 1:

Abdominal subcutaneous fat gene expression and circulating levels of leptin and adiponectin in polycystic ovary syndrome

Abdominal subcutaneous fat gene expression and circulating levels of leptin and adiponectin in polycystic ovary syndrome

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Conflicts of interest: none to declare
Financial support: This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq INCT 573747/2008-3), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES 1289/05) and Fundo de Apoio à Pesquisa do Hospital de Clínicas de Porto Alegre (FIPE-HCPA 340/2004), Brazil.

Capsule: In PCOS, leptin:adiponectin ratio was a marker of altered adipocyte secretion, which was related to adiposity, not androgen excess. Serum adipokine levels correlated with gene expression in fat tissue.

ABSTRACT

Objectives. To determine: 1) leptin and adiponectin serum levels and gene expression in subcutaneous adipose tissue from polycystic ovary syndrome (PCOS) and non-hirsute, ovulatory women; 2) leptin to adiponectin (L/A) ratio.

Design. Case-control study.

Setting. University Hospital Gynecological Endocrinology Unit.

Patients. 31 PCOS women, 59 controls.

Interventions. Anthropometric, hormonal, and metabolic assessment; subcutaneous adipose tissue biopsy.

Main outcome measures. Leptin and adiponectin serum levels and gene expression; L/A ratio in PCOS and controls, stratified by BMI.

Results. Serum leptin was higher in overweight/obese PCOS patients than in all normoweight women (P <0.001). Adiponectin levels were similar in all subgroups. L/A ratio was higher in overweight/obese subgroups than in normoweight controls [5.27 (2.66-13.58) and 7.73 (3.81-15.04) vs. 1.80 (0.94-3.72); P <0.001]. Subcutaneous leptin mRNA was higher in overweight/obese PCOS women than in normoweight controls [2.316 (1.987-2.580) vs. 1.687 (1.518-2.212), P = 0.038]. Adiponectin gene expression was similar in all groups (P = 0.272). On multiple regression analysis, percentage of body fat contributed significantly to L/A ratio in PCOS, independently of BMI and free androgen index.

Conclusions. In PCOS, altered adipocyte secretion seems to relate to adiposity rather than to androgen excess.

Key words: adipokines, PCR-real time, PCOS, adipose tissue, insulin resistance, central obesity

INTRODUCTION

Adipose tissue has been recognized as an active endocrine organ playing a pleiotropic role. Adipocyte-secreted proteins known as adipokines have been proposed as mediators for the central obesity, insulin sensitivity and other components characterizing the metabolic syndrome. Two adipokines, leptin and adiponectin have been associated with body mass index (BMI), insulin action, and glucose metabolism. However, different or complementary mechanisms seem to be involved in leptin or adiponectin regulation of metabolic homeostasis (1,2).

Obesity (predominantly abdominal) is observed in 30 to 75% of women with PCOS (3-6). In these patients, elevated serum leptin levels are associated with obesity and metabolic disturbances (7-10). In contrast, conflicting results have been reported for adiponectin: some groups have observed decreased levels of adiponectin in PCOS patients compared with weight- and BMI–matched controls (9,11-15), while others report no difference between PCOS and controls after controlling for obesity (16-18). Xita et al. have proposed that leptin to adiponectin ratio (L/A ratio), rather than adiponectin or leptin alone, relates to insulin resistance in women with PCOS (19).

Leptin gene expression in subcutaneous adipose tissue is also positively correlated with BMI in obese subjects (20-23). For adiponectin gene expression, an inverse correlation with BMI, obesity, and metabolic risk factors has been reported by some investigators (20,24-27), but not by others (22,28). The few studies investigating adipokine gene expression in adipose tissue from PCOS women show lower leptin and adiponectin expression (13).

Therefore, the aims of the present study were to characterize leptin and adiponectin gene expression in subcutaneous adipose tissue from PCOS and non-hirsute, ovulatory control women, as well as to determine the circulating levels of these adipokines and the leptin to adiponectin (L/A) ratio. We also aimed to establish whether there is an association between leptin and adiponectin features and adiposity, androgens, and insulin resistance.

MATERIALS AND METHODS

Patients and controls

This case-control study was carried out with women at reproductive age consulting at the Gynecological Endocrinology Unit at Hospital de Clínicas de Porto Alegre, Brazil. Women with BMI ranging from 18.5 to 39.9 kg/m² were selected for the study. Thirty-one hirsute women presenting oligo/amenorrheic cycles (<9/year), increased levels of serum testosterone and/or free androgen index (FAI), and absence of other disorders causing hirsutism (8,29) were enrolled. A control group was set up with 59 non-hirsute women with regular, ovulatory cycles (luteal phase progesterone levels >3.8 ng/mL). Transabdominal or transvaginal ovarian ultrasound was performed in all patients. None of the participants had received any drugs known to interfere with hormonal levels for at least 3 months before the study. Women with diabetes, liver or renal disease, thyroid dysfunction or pregnancy were excluded. The study protocol was approved by the local Ethics Committee (Institutional Review Board-equivalent), and written informed consent was obtained from all subjects.

Study protocol

Medical interview and physical examination were performed as described (30,31). Hirsutism was defined as a modified Ferriman–Gallwey score ≥8 (32). Blood pressure was measured after a 10-min rest period (33). Anthropometric measurements included body weight, height, body mass index (BMI), waist circumference (30,34), hip circumference (35), waist to hip ratio (WHR) and percentage of body fat, calculated by the sum of triceps, subscapular, suprailiac, and abdominal skinfolds (36). Skinfold thickness was estimated in triplicate with a caliper (Cescorf, Mitutoyo, Porto Alegre, Brazil) with 0.1 mm scale and pressure of 10 g/mm^2 (37).

Hormonal and metabolic assessment was performed between days 2 and 10 of the menstrual cycle or on any day if the patient was amenorrheic. All samples were obtained between 8 and 10 a.m. After an overnight 12-hour fast, blood samples were drawn from an antecubital vein for determination of plasma leptin, adiponectin, and lipid profile (total cholesterol, HDL cholesterol and triglycerides) at baseline and glucose and insulin before and 2 hours after the ingestion of 75 g of oral anhydrous glucose (OGTT). Blood samples were also assessed for measurement of highly sensitive C-reative protein (hs-CRP), estradiol, luteinizing hormone (LH), sex hormone binding globulin (SHBG), and total testosterone (TT). Free androgen index (FAI) was estimated

by dividing TT (nmol/L) by SHBG (nmol/L) x 100. L/A ratio was calculated by dividing serum leptin by serum adiponectin concentrations. Homeostasis model assessment index to estimate insulin resistance (HOMA-IR) was calculated by multiplying insulin (μIU/mL) by glucose (mmol/L) and dividing this product by 22.5 (38). LDL cholesterol was estimated indirectly with the Friedewald formula (39). Lipid acummulation product (LAP) index was calculated using the formula [waist circumference (cm) - 58] x triglycerides (mmol/L) (31). A control subsample (n = 37) underwent complete clinical, anthropometric and metabolic evaluation and the OGTT.

Biochemical and Hormonal Assays

Total cholesterol, HDL cholesterol, triglycerides and glucose were determined by colorimetric-enzymatic methods. Serum leptin and total adiponectin concentrations were measured using commercially available ELISAs (LINCO Research, Missouri, USA). Serum hs-CRP concentrations were measured using the nephelometry method (Siemens Dade Behring, Germany), with intra- and interassay coefficient of variation (CV) <5%. Serum insulin, estradiol, LH and SHBG levels were measured with electrochemiluminescent immunoassays (Roche Diagnostic, Mannhein, Germany), with intra-assay CV <3%, and interassay CV <5%. Total serum testosterone levels were measured with the radioimmunoassay method (Diagnostics Systems Laboratory— DSL, Texas, USA) with intra- and interassay CV <9.6%.

Tissue collection

Adipose tissue biopsy was performed to obtain a 250 mg periumbilical sample of subcutaneous fat. The procedure was performed between days 2 and 10 of the menstrual cycle or on any day if the patient was amenorrheic, by surgeons from the Plastic Surgery Service. All participants were submitted to standard local anesthetic procedures. Subcutaneous fat fragments were immediately frozen in liquid nitrogen and stored at -80°C until total RNA isolation.

RNA isolation

Adipose tissue total RNA was isolated and analyzed (40,41). Subcutaneous adipose tissue fragments were homogenized in phenol-guanidine isothiocyanate (Trizol®, Invitrogen™ Life Technologies, USA). Total RNA was extracted with

chloroform and precipitated with isopropanol by centrifugation (12,000 × g) at 4°C. The RNA pellet was washed twice with 75% ethanol and resuspended in diethylpyrocarbonate (DEPC) treated water. Concentration and quality of isolated total RNA were assessed using GeneQuant spectrophotometer (Pharmacia Biotech, Cambridge, England).

Real time RT-PCR protocol

Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed in two separate reactions: first, total RNA was reverse transcribed into cDNA, then the cDNA was amplified by real-time PCR. Reverse transcription of 1 µg of total RNA into cDNA was carried out using the Superscript II First-Strand Synthesis System for RT-PCR (Invitrogen™ Life Technologies, USA), according to the manufacturer's protocol, and in a PCT-100™ Programmable Thermal Controller (MJ Research Inc., USA). The RNA template was initially denatured at 65°C for 5 min with a mixture of dNTPs (10 mM each dATP, dCTP, dGTP, dTTP). The annealing of oligo $(dT)_{12-18}$ (0.5 µg/µL) was performed at 42°C for 2 min with the addition of a mixture of 10X RT buffer (200 mM Tris-HCl pH 8.4; 500 mM KCl), MgCl2 (25 mM), DTT (0.1 M) and recombinant RNase inhibitor (40 U/µL RNaseOUTTM). One microliter of SuperscriptTM II RT (50 U/µL) was added to each reaction tube and cDNA synthesis was performed during 50 min at 42°C. The mixture was heated at 70°C for 15 min to interrupt the reaction. Each cDNA sample was purified with 1 µL RNase H (2 U/µL) for 20 min at 37°C. RT-PCR was carried out in a final volume of 23 µL. cDNA samples were stored at -20°C until gene expression amplification.

Real-time PCR experiments were carried out in a 7500 Fast Real-Time PCR System thermal cycler with 7500 Fast System Sequence Detection 1.4 Software (Applied Biosystems, USA). Real-time PCR was performed by monitoring in real time the increase in fluorescence of the SYBR® Green dye as previously described (42-44). Primers were designed and optimized with regard to primer dimers, self priming, miss priming and amplicon length using the Primer Express 3.0 Software for Real-Time PCR (Applied Biosystems, USA) and obtained from Invitrogen™ Life Technologies, USA. The forward and reverse primer sequences were projected to target two exons of an mRNA in order to prevent the amplification of any contaminating genomic DNA and with respect to known splice variants and single-nucleotide polymorphism positions

documented. The forward and reverse primer sequences designed for leptin (NM_000230.2, *H. sapiens* leptin (LEP), mRNA) were (5' to 3') TCCCCTCTTGACCCATCTC and GGGAACCTTGTTCTGGTCAT, respectively. These primers anneal between residues 858 to 876 (forward) and 967 to 948 (reverse), producing a PCR product of 110 bp. The forward and reverse primer sequences for adiponectin (NM_004797.2, *H. sapiens* adiponectin, C1Q and collagen domain containing (ADIPOQ), mRNA) were (5' to 3') TGGAGTGTTGGTAGGTGTCTGT and GGGCCATTGAGTAGTAGTCCTT, respectively. These primers anneal between residues 1,280 to 1,301 (forward) and 1,431 to 1,410 (reverse) and produce an amplicon of 152 bp. Glyceraldehyde-3-phosphate dehydrogenase (NM_002046.3, *H. sapiens* glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA) was used to normalize mRNA quantitation. ACCCACTCCTCCACCTTTG and CTCTTGTGCTCTTGCTGGG (5' to 3'), respectively, forward and reverse GAPDH primers sequences, anneal between residues 970 to 988 (forward) and 1,147 to 1,129 (reverse), resulting in an amplicon of 178 bp. cDNA samples were diluted to a final concentration of 0.25 ng/µL and mixed with a predetermined forward and reverse primer volume (respectively, 0.9 and 0.7 µL for leptin, 0.3 and 0.7 µL for adiponectin, 0.9 and 0.9 µL for GAPDH) and 12.5 µL of 2X Fast SYBR® Green Master Mix (Applied Biosystems, USA) in a total volume of 25 µL loaded per sample on the MicroAmp™ Fast Optical 96-Well Reaction Plate sealed with MicroAmp™ Optical Adhesive Film (Applied Biosystems, USA). Final real-time PCR protocol conditions consisted of denaturation at 94°C for 2 min followed by 50 cycles (30 sec at 94°C and 30 sec at 60°C) for leptin or by 50 cycles (30 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C) for adiponectin. Primers generated amplicons that produced a single sharp peak during melting curve analysis. All PCR reactions were performed in triplicate.

Data analysis from leptin and adiponectin real-time PCRs was performed by relative quantitation using the comparative C_T method as described (45). Validation assays were performed by amplification of the target and reference genes, separately, using serial dilutions of an mRNA sample. Both target and reference mRNAs presented equal efficiencies of amplification. The comparative C_T method calculates changes in gene expression as relative fold difference between an experimental and an external calibrator sample, including a correction for non-ideal amplification efficiencies (46).

Statistical analysis

Data were described as mean ± SD or median and interquartile range (25% to 75%). Comparisons between means were analyzed by the unpaired two-tailed Student's *t* test or Mann-Whitney *U* test. Comparisons between group means were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey *post hoc* test. Pearson's or Spearman's rank correlations coefficients were calculated between variables using a two-tailed test for significance. A forward stepwise multiple regression model was also calculated for patients with PCOS using the L/A ratio as a dependent variable and BMI, percentage of body fat and FAI as independent variables. Log_{10} transformation was used to normalize the distribution of non-Gaussian variables and mean values were back-transformed for presentation. Data were considered statistically significant at $P < 0.05$. The Statistical Package for the Social Sciences 16 (SPSS, Chicago, IL, USA) was used in the analysis.

RESULTS

Table 1 summarizes the clinical, metabolic and hormonal profiles of control and PCOS women. The two groups had similar BMI (P = 0.110). As expected, hirsutism score, androgen levels, FAI and LH were higher with PCOS (P <0.001). Two-hour glucose levels were strikingly higher in PCOS. PCOS patients were younger than controls (P <0.001), but presented hyperinsulinemia, as indicated by HOMA-IR and insulin.

Table 2 shows data stratified according to BMI (<25 kg/m^2 or ≥25 kg/m^2). Serum leptin concentrations, waist circumference, percentage of body fat and LAP were significantly higher in overweight/obese PCOS patients than in normoweight subgroups (with or without PCOS) (P <0.001); however, these parameters were similar in overweight/obese subgroups. WHR and HOMA-IR were higher in overweight/obese PCOS patients in comparison to all the other subgroups. Although adiponectin serum levels were similar in all groups, L/A ratio was strikingly higher in overweight/obese control and PCOS women than in normoweight controls [5.27 (2.66-13.58) and 7.73 (3.81-15.04) vs. 1.80 (0.94-3.72) respectively, P <0.001] (Figure 1A).

Subcutaneous leptin mRNA was significantly higher in overweight/obese PCOS women than in normoweight controls [2.316 (1.987-2.580) vs. 1.687 (1.518-2.212) n fold change in relation to the calibrator sample, respectively, P = 0.038] (Figure 1B). Adiponectin gene expression was similar in all subgroups (P = 0.272) (Figure 1C).

L/A ratio was positively correlated with BMI and negatively with SHBG in controls (Table 3). In PCOS, L/A ratio was correlated with BMI, waist circumference, percentage of body fat, fasting insulin, post-prandial glucose and insulin levels, HOMA-IR, LH concentration and FAI. A stepwise regression model revealed that percentage of body fat contributed positively and significantly to L/A ratio in PCOS, independently of BMI or FAI (Table 3).

DISCUSSION

In the present study, leptin gene expression and levels were higher in obese PCOS than in normoweight controls, but no differences were observed regarding adiponectin. Subcutaneous adipose tissue production of adiponectin seems to be the main determinant of serum adiponectin concentrations. Our data agree with previous reports referring to non-PCOS populations (20,21,25), but differ from the results of one previous work reporting lower leptin and adiponectin gene expression in subcutaneous fat of PCOS women vs. weight-matched controls (13). Ethnic variation could explain this difference: while we studied South Brazilian samples, Carmina et al. studied American women with PCOS and Mexican controls.

Leptin gene expression in subcutaneous adipose tissue was higher in our overweight/obese PCOS patients as compared to normoweight controls, but not to normoweight PCOS and overweight/obese controls. Other authors have shown that leptin mRNA expression in subcutaneous fat was significantly elevated in the presence of obesity (20,22,23). In addition, there is evidence that subcutaneous fat leptin may be partially regulated at the transcriptional level during energy restriction (21,47-49).

We chose to measure leptin and adiponectin gene expression strictly in subcutaneous fat. Despite the regional variation in leptin and adiponectin expression in different types of adipose tissue, expression of both adipokines are higher in subcutaneous than in visceral fat. In fact, adiponectin and leptin are predominantly produced by subcutaneous adipocytes in obese women (26,50-52). Another advantage of using subcutaneous fat is that the procedure for obtaining samples is less invasive than for visceral fat. All our PCOS and control participants agreed to be submitted to abdominal adipose tissue biopsy, and we did not have to rely on volunteers being submitted to a variety of elective surgical procedures, a limitation of previous studies.

Circulating leptin levels are strongly influenced by abdominal fat content and distribution (9,12,20,26). Our overweight/obese PCOS and control participants showed higher waist circumference, percentage of body fat and serum leptin levels than normoweight participants, confirming that serum leptin concentration is linked to the amount of adipose tissue and BMI, as described (12,20,26). Furthermore, serum leptin levels in PCOS were associated with overall and central adiposity, and not directly with PCOS itself, as previously observed by our group (8) and others (7,9,10).

Many investigators have examined the relationship between circulating adiponectin levels and PCOS features, yet with conflicting results. A meta-analysis (53)

found that in studies with a small number of participants, or including PCOS women who were markedly more insulin-resistant than controls, total adiponectin serum concentration was significantly lower in PCOS patients. In contrast, in larger studies, and/or in studies with groups presenting only slight or moderate degrees of IR, which is closer to the present investigation, total serum adiponectin was similar to that of controls. Recent evidence suggests that adiponectin multimers with different molecular weights occur in the circulation, but that only high molecular weight (HMW) multimers are active; these active adiponectin forms would be more useful than total adiponectin to evaluate insulin sensitivity and glucose tolerance (54,55) in PCOS (56).

Other recent studies indicate that L/A ratio may be highly correlated with IR, cardiovascular risk factors, and with development of the metabolic syndrome (19,20,57,58). In our study, L/A ratio was positively associated with HOMA-IR. Interestingly, the positive correlation observed between L/A ratio and FAI was dependent on BMI. In contrast, the androgen-independent correlation between L/A ratio and percentage of body fat supports the relevance of body fat content and distribution for adypocite dysfunction in PCOS. These data support the notion that L/A ratio is indeed a more sensitive marker of adipocyte secretion than isolated leptin and adiponectin circulating levels.

In conclusion, altered adipocyte secretion seems to be more related to adiposity than to androgen excess in PCOS. Further studies are required to investigate whether calorie restriction and weight loss correlate with changes in leptin and adiponectin gene expression in PCOS, and whether these putative changes could be associated with decreased prevalence of cardiovascular risk factors.

ACKNOWLEDGMENTS

We thank Dr. Rinaldo de Angeli Pinto and his staff of the Plastic Surgery Service at Hospital de Clínicas de Porto Alegre, RS, Brazil, for performing the biopsies of periumbilical adipose tissue.

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Table 1. Clinical, metabolic and hormonal features of control and PCOS women

Characteristic	Control (n = 59)	PCOS (n = 31)	P
Age (years)	31.5 ± 5.3	23.9 ± 5.9	**<0.001**
BMI (kg/m^2)	28 ± 5	30 ± 6	0.110
Ferriman-Gallwey score	2 (0-5)[a]	9 (8-14)	**<0.001**
Systolic BP (mmHg)	117 ± 10[a]	118 ± 16	0.742
Diastolic BP (mmHg)	75 ± 10[a]	74 ± 11	0.578
Fasting glucose (mg/dL)	90 ± 8[a]	90 ± 7	0.937
Glucose 2h (mg/dL)	97 ± 19[a]	115 ± 28	**0.002**
Fasting insulin (uIU/mL)	9 (4-13)	12 (8-18)	**0.003**
Insulin 2h (uIU/mL)	46 (28-82)[a]	82 (46-173)	**0.002**
Triglycerides (mg/dL)	63 (46-99)	78 (49-111)	0.330
Total cholesterol (mg/dL)	156 ± 40	171 ± 40	0.114
HDL cholesterol (mg/dL)	49 ± 17	51 ± 10	0.567
LDL cholesterol (mg/dL)	92 ± 31	104 ± 35	0.110
hs-CRP (mg/L)	1.74 (0.73-3.74)[a]	4.31 (0.75-10.90)	**0.031**
LH (mIU/mL)	5.41 (3.72-7.46)[a]	11.23 (7.42-15.46)	**<0.001**
SHBG (nmol/L)	44.10 (26.15-57.93)	32.90 (18.88-47.93)	0.069
Total testosterone (ng/mL)	0.58 (0.49-0.67)	1.18 (0.88-1.47)	**<0.001**
FAI	4.52 (3.51-6.50)	11.36 (6.83-21.29)	**<0.001**
Estradiol (pg/mL)	51.2 (33.0-106.7)	55.0 (39.7-68.3)	0.806

BP: blood pressure; BMI: body mass index; FAI: free androgen index; hs-CRP: highly sensitive C-reative protein; HDL: high-density lipoprotein; LDL: low-density lipoprotein; LH: luteinizing hormone; PCOS: polycystic ovary syndrome; SHBG: sex hormone binding globulin. Values are expressed as mean ± SD (Student's *t* test) or median and interquartile range (25% to 75%) (Mann-Whitney *U* test).

[a]n = 37.

Table 2. Adipokines serum levels, anthropometric and metabolic variables for control and PCOS women stratified according to body mass index (BMI)*

	Control		PCOS		
	BMI <25 (n = 19)	BMI ≥25 (n = 38)	BMI <25 (n = 8)	BMI ≥25 (n = 23)	P
Waist circumference (cm)	†72.9 ± 6.5[a]	††88.8 ± 8.3[b]	†74.0 ± 7.0	††95.1 ± 9.8	**<0.001**
WHR	†0.74 ± 0.04[a]	†0.78 ± 0.05[b]	†0.74 ± 0.04	††0.85 ± 0.08	**<0.001**
Body fat (%)	†18.7 ± 5.2[a]	††28.9 ± 4.1[b]	†19.6 ± 2.8	††31.4 ± 4.6	**<0.001**
LAP	†8.45 (5.25-9.15)[a]	††25.12 (14.41-40.28)[b]	†8.52 (6.24-17.41)	††38.43 (21.99-50.73)	**<0.001**
HOMA-IR	†1.0 (0.8-1.7)[a]	††2.4 (1.6-3.2)[b]	†1.5 (0.8-1.7)	†††3.4 (2.3-4.3)	**<0.001**
Serum leptin (ng/mL)	†8.61 (5.12-13.73)	††27.39 (21.46-34.57)	†12.47 (5.87-16.57)	††36.78 (27.38-52.57)	**<0.001**
Serum adiponectin (ng/mL)	4.34 (2.36-5.88)	3.98 (1.77-8.23)	4.33 (3.31-5.98)	4.46 (2.96-8.40)	0.774

HOMA-IR: homeostasis model assessment; LAP: lipid accumulation product; PCOS: polycystic ovary syndrome; WHR: waist to hip ratio. Values are expressed as *kg/m^2, mean ± SD or median and interquartile range (25% to 75%).

[a] n = 7.

[b] n = 28.

Different symbols indicate statistical difference by One-way ANOVA + Tukey *post-hoc* test.

Table 3. Correlation of anthropometric, metabolic and hormonal profiles with serum leptin to adiponectin ratio and model-fitting results for stepwise regression of serum leptin to adiponectin ratio vs. body mass index, percentage of body fat and free androgen index with significantly correlated variables for PCOS

L/A ratio vs.	Control		PCOS	
	R	P	R	P
BMI	0.441	**0.001**	0.522	**0.003**
Waist circumference	0.312	0.077	0.450	**0.011**
Body fat (%)	0.344	0.050	0.714	**<0.001**
Fasting glucose	0.064	0.721	-0.019	0.917
Glucose 2h	-0.123	0.487	0.410	**0.022**
Fasting insulin	0.255	0.063	0.402	**0.025**
Insulin 2h	0.062	0.727	0.412	**0.021**
HOMA-IR	0.199	0.259	0.387	**0.032**
SHBG	-0.316	**0.019**	-0.206	0.267
Total testosterone	0.111	0.454	0.282	0.138
FAI	0.158	0.283	0.375	**0.045**

L/A ratio vs. independent variables	Coefficient ± SE	P
BMI	-0.037 ± 0.022	0.114
Body fat (%)	0.080 ± 0.021	**0.001**
FAI	-0.175 ± 0.202	0.395

BMI: body mass index; FAI: free androgen index; HOMA-IR: homeostasis model assessment; L/A ratio: serum leptin to adiponectin ratio; SHBG: sex hormone binding globulin. Values for leptin to adiponectin ratio and FAI were $\log_{10}$ transformed.

Figure 1.

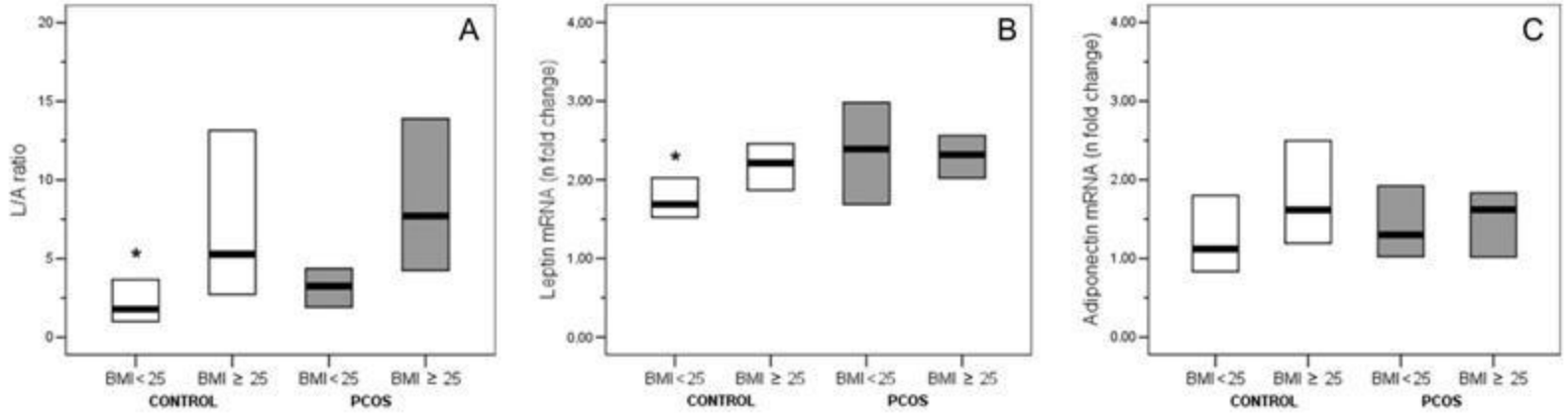
A
L/A ratio
20
15
10
5
0
*
BMI < 25
BMI ≥ 25
BMI < 25
BMI ≥ 25
CONTROL
PCOS
B
Leptin mRNA (n fold change)
4,00
3,00
2,00
1,00
0,00
*
BMI < 25
BMI ≥ 25
BMI < 25
BMI ≥ 25
CONTROL
PCOS
C
Adiponectin mRNA (n fold change)
4,00
3,00
2,00
1,00
0,00
BMI < 25
BMI ≥ 25
BMI < 25
BMI ≥ 25
CONTROL
PCOS

FIGURE LEGENDS

Figure 1. A) Serum leptin to adiponectin ratio (L/A ratio) for control and PCOS women stratified according to body mass index (kg/m^2). Values are expressed as median and interquartile range (25% to 75%). One-Way ANOVA + Tukey *post hoc* test. *P<0.001 in relation to overweight/obese control and PCOS groups. B) Leptin and C) adiponectin gene expression in subcutaneous adipose tissue from control and PCOS women stratified according to body mass index (kg/m^2). Messenger ribonucleic acid (mRNA) was expressed as n fold change differences to the calibrator sample ($\Delta\Delta C_T$ method). Values are expressed as median and interquartile range (25% to 75%). One-way ANOVA + Tukey *post hoc* test. *P = 0.038 in relation to overweight/obese PCOS group.

Parte III

Artigo original 2:

Association between blood pressure and CYP19 gene expression in subcutaneous adipose tissue of women with polycystic ovary syndrome

Association between blood pressure and CYP19 gene expression in subcutaneous adipose tissue of women with polycystic ovary syndrome

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ABSTRACT

Introduction: The activity of aromatase, an enzyme encoded by the CYP19 gene, affects androgen metabolism and estrogen synthesis, as well as the androgen-to-estrogen balance in different tissues (1, 2). This study characterized CYP19 gene expression in subcutaneous adipose tissue from normotensive and hypertensive patients with polycystic ovary syndrome (PCOS) and evaluated whether subcutaneous fat CYP19 mRNA is associated with androgen and estrogen levels and blood pressure (BP) in PCOS.

Methods: This case-control study included 31 PCOS women of reproductive age and 27 normotensive non-hirsute controls. Hypertension was defined as BP ≥130/85 mmHg (3). Metabolic and hormonal measurements were made. Real-time quantitative PCR determined gene expression levels ($\Delta\Delta C_T$ Method).

Results: Table 1 shows clinical and hormonal results. CYP19 mRNA in subcutaneous fat from PCOS patients correlated positively with systolic (r=0.509 p=0.006) and diastolic (r=0.485 p=0.009) BP.

Discussion: Our data suggest that androgen excess and hyperinsulinemia may play a role on the molecular mechanisms that activate aromatase mRNA transcription in abdominal fat tissue in women with PCOS.

Key words: blood pressure; CYP19 mRNA; subcutaneous adipose tissue; PCOS

INTRODUCTION

Polycystic ovary syndrome (PCOS), the most prevalent endocrine disorder in women of reproductive age, affects 5 to 10% of women worldwide (4, 5). PCOS is characterized by hyperandrogenism, ovulatory dysfunction, and polycystic ovaries in its complete phenotype (6-12) in the absence of other diseases of the pituitary and/or adrenal glands (6, 11, 13, 14). Along with androgen excess, women with PCOS are more likely than normally cycling women to have insulin resistance, central adiposity, dyslipidemia, and hypertension (4, 5, 15-22). These factors increase the risk for cardiovascular events in women with PCOS (21, 23-26).

Adipose tissue has been established as an active endocrine organ and a secretory mediator of numerous physiological processes. The evaluation of its pleiotropic functions reveals that adipose tissue is a reservoir of steroids, a center of sexual hormone conversion and a source of estrogen in both men and women. Estrogen biosynthesis is catalyzed by aromatase, an enzyme encoded by the CYP19 gene that belongs to the cytochrome P450 superfamily, which is responsible for the synthesis of cholesterol and steroid hormones and for the metabolism of fatty acids. Aromatase catalyzes a complex reaction sequence that results in the conversion of androgens (C19), namely testosterone and androstenedione, into two estrogens (C18), estradiol and estrone (27). In fact, aromatase activity affects both androgen metabolism and estrogen synthesis. Therefore, the biological importance of this enzyme arises not only from its role in estrogen production, but also from its potential effect on the androgen-to-estrogen balance in different tissues (1, 2).

The association between estrogens and cardiovascular disease in general and hypertension in particular has been studied extensively in both experimental and human models. Aromatase-deficient (ArKO) female mice that were deficient in estrogens due to deletion of the CYP19 gene had lower blood pressure (BP) levels, which suggests that aromatase may be one of the factors that affect BP and CYP19, a susceptibility gene for hypertension (28). Although the associations between genes involved in estrogen action and hypertension remain to be completely understood, several studies suggest that estrogen-related genes and sex hormone levels affect BP regulation (29-32).

Therefore, this study characterized CYP19 gene expression in subcutaneous adipose tissue of women with PCOS and in a control group of non-hirsute, ovulatory women. We also evaluated the association between subcutaneous fat CYP19 mRNA

and hormone circulating levels and BP in normotensive and hypertensive women with PCOS.

MATERIALS AND METHODS

Patients and controls

This case-control study enrolled women of reproductive age seen at the Gynecological Endocrinology Unit of Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil. Women with a body mass index (BMI) ranging from 18.5 to 39.9 kg/m² were selected for the study. Thirty-one hirsute oligo-amenorrheic women (<9 cycles/year) were enrolled. They had increased serum testosterone levels and/or free androgen index (FAI) and no other disorders causing hirsutism (6, 11, 13, 14). The control group enrolled 27 normotensive non-hirsute women with regular ovulatory cycles (luteal phase progesterone levels higher than 3.8 ng/ml). Abdominal or transvaginal ovarian ultrasound was performed in all patients. None of the women from either group had received any drugs known to interfere with hormone levels for at least 3 months before the study. Women with diabetes, liver or renal disease, thyroid dysfunction or pregnancy were excluded. The study protocol was approved by the local Ethics Committee (Institutional Review Board-equivalent), and written informed consent was obtained from all participants.

Study protocol

A medical interview and physical examination were performed according to standard protocols as previously described (6, 14, 33-35). Hirsutism was defined as a modified Ferriman–Gallwey score of 8 or more (36). Blood pressure was measured in the supine position after a 10-minute rest. Hypertension was defined as systolic blood pressure (SBP) ≥130 mmHg and/or diastolic blood pressure (DBP) ≥85 mmHg (3).

Anthropometric measurements were body weight, height, body mass index (current weight in kg divided by height in m^2) and percentage of body fat. Percentage of body fat was calculated by the sum of triceps, subscapular, suprailiac and abdominal skin folds (37). Skin fold thickness was estimated in triplicate with a caliper (Cescorf, Mitutoyo, Porto Alegre, Brazil) with a 0.1 mm scale and pressure of 10 g/mm^2 (38).

Hormonal and metabolic assessment was performed between days 2 and 10 of the menstrual cycle or on any day if the patient was amenorrheic. All samples were obtained between 8 and 10 a.m. After an overnight 12-hour fast, blood samples were drawn from an antecubital vein for measurement of serum glucose, insulin, luteinizing hormone (LH), follicle stimulating hormone (FSH), sex hormone binding globulin (SHBG), 17-hydroxyprogesterone (17OHP), dehydroepiandrosterone sulfate (DHEAS),

total testosterone (TT), estradiol (E_2) and highly sensitive C-reactive protein (hs-CRP). Blood samples were also used to determine plasma fibrinogen and white blood cell (WBC) counts. The free androgen index (FAI) was calculated as the ratio between TT and SHBG serum levels. The estrogen-to-androgen ratio was calculated by dividing E_2 by TT serum concentrations. Estrogen-to-androgen ratio was also categorized as ≤ or > 0.06 (median estrogen-to-androgen ratio for the three groups: controls, hypertensive PCOS and normotensive PCOS). Homeostasis model assessment index to estimate insulin resistance (HOMA-IR) was calculated by multiplying insulin (μIU/mL) by glucose (mmol/L) and dividing it by 22.5 (39, 40). The cut-off point to determine insulin resistance (IR) was arbitrarily defined as a HOMA-IR ≥3.8 (34).

Assays

Serum glucose was determined using a colorimetric-enzymatic method (Advia System – Siemens, Germany). Insulin, LH, FSH, SHBG, DHEAS and E_2 serum concentrations were measured with electrochemiluminescent immunoassays (Roche Diagnostic, Germany) with an intra-assay coefficient of variation (CV) of <3% and interassay CV of <5%. Total testosterone and 17OHP serum levels were measured using radioimmunoassays (DSL, Texas, USA and ICN Biomedicals Inc., Costa Mesa, USA, respectively) with an intra- and interassay CV of <9.6% for TT and of <12.9% for 17OHP. Serum hs-CRP concentrations were determined using the nephelometry method (Dade Behring – Siemens, Germany), which has a CV of <5%. Fibrinogen was measured using a coagulometer (Dade Behring – Siemens, Germany), with a CV of 5%. WBC count was analyzed using light absorbance, impedance and flow cytometry (Roche Diagnostics, Germany).

Tissue collection

Adipose tissue biopsy was performed in 47 individuals (19 controls and 28 PCOS women) to obtain a 250 mg sample of subcutaneous fat from the periumbilical region. The procedure was performed between days 2 and 10 of the menstrual cycle or on any day if the patient was amenorrheic, by surgeons from the Plastic Surgery Service at Hospital de Clínicas de Porto Alegre. All participants received the same local anesthetic procedures, and the subcutaneous fat fragments were immediately frozen in liquid nitrogen and stored at -80°C until total RNA isolation.

RNA isolation

White adipose tissue total RNA was isolated and analyzed as previously described (41, 42). Subcutaneous adipose tissue fragments were homogenized in phenol-guanidine isothiocyanate (Trizol®, Invitrogen™ Life Technologies, Foster City, USA). Total RNA was extracted with chloroform and precipitated with isopropanol by centrifugation (12,000 × g) at 4°C. The RNA pellet was washed twice with 75% ethanol and resuspended in water treated with diethylpyrocarbonate (DEPC). Concentration and quality of isolated total RNA were assessed using a GeneQuant spectrophotometer (Pharmacia Biotech, Cambridge, England).

Real time RT-PCR protocol

Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed in two separate reactions: first, total RNA was reverse transcribed into cDNA, then the cDNA was amplified using real-time PCR. Reverse transcription of 1 µg of total RNA into cDNA was carried out using the Superscript II First-Strand Synthesis System for RT-PCR (Invitrogen™ Life Technologies, Foster City, USA) according to the manufacturer's protocol, in a PCT-100™ programmable thermal controller (MJ Research Inc., USA). The RNA template was initially denatured at 65° C for 5 min with a mixture of dNTPs (10 mM each dATP, dCTP, dGTP, dTTP). The annealing of oligo $(dT)_{12-18}$ (0.5 µg/µL) was performed at 42° C for 2 min with the addition of a mixture of 10X RT buffer (200 mM Tris-HCl pH 8.4; 500 mM KCl), $MgCl_2$ (25 mM), DTT (0.1 M) and recombinant RNase inhibitor (40 U/µL RNaseOUT™). One microliter of Superscript™ II RT (50 U/µL) was added to each reaction tube and cDNA synthesis was performed during 50 min at 42° C. The mixture was heated to 70° C for 15 min to interrupt the reaction. Each cDNA sample was purified with 1 µL RNase H (2 U/µL) for 20 min at 37° C. RT-PCR was carried out in a final volume of 23 µL. cDNA samples were stored at -20° C until gene expression amplification.

Real-time PCR experiments were carried out in a 7500 Fast Real-Time PCR System thermal cycler and the 7500 Fast System Sequence Detection 1.4 Software (Applied Biosystems, Foster City, USA). Real-time PCR was performed by monitoring in real time the increase in fluorescence of the SYBR® Green dye as previously described (43-45). Primers were designed and optimized for primer dimers, self-

priming, mispriming and amplicon length using the Primer Express 3.0 Software for Real-Time PCR (Applied Biosystems, Foster City, USA) and acquired using the Invitrogen™ (Life Technologies, Foster City, USA). The forward and reverse primer sequences were projected to target two exons of an mRNA to prevent the amplification of any contaminating genomic DNA and for known splice variants and single-nucleotide polymorphism positions documented. The forward and reverse primer sequences designed for CYP19 (NM_ 000103.3 and NM_031226.2, *H. sapiens* cytochrome P450, family 19, subfamily A, polypeptide 1 (CYP19A1), transcript variant 1 and 2, mRNAs) were (5' to 3') AACGTGGTGACCTGACAAGA and TCTTCAACATTAGGGTGCTTTG. These primers anneal between residues 1,002 to 1,021 (forward) and 1,131 to 1,110 (reverse) for transcript variant 1 and between residues 1,111 to 1,130 (forward) and 1,240 to 1,219 (reverse) for transcript variant 2. Both variants encode the same protein, and thus produce a PCR product of 130 bp. Glyceraldehyde-3-phosphate dehydrogenase (NM_002046.3, *H. sapiens* glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA) was used to normalize mRNA quantitation. ACCCACTCCTCCACCTTTG and CTCTTGTGCTCTTGCTGGG (5' to 3'), forward and reverse GAPDH primer sequences, anneal between residues 970 to 988 (forward) and 1,147 to 1,129 (reverse), resulting in an amplicon of 178 bp. cDNA samples were diluted to a final concentration of 0.50 ng/µL and mixed with a predetermined forward and reverse primer volume (0.7 and 0.9 µL for CYP19; 0.9 and 0.9 µL for GAPDH) and 12.5 µL of 2X Fast SYBR® Green Master Mix (Applied Biosystems, Foster City, USA) in a total volume of 25 µL loaded per sample on the MicroAmp™ Fast Optical 96-Well Reaction Plate sealed with MicroAmp™ Optical Adhesive Film (Applied Biosystems, Foster City, USA). The real-time PCR protocol consisted of denaturation at 94° C for 2 min followed by 45 cycles (30 sec at 94° C, 30 sec at 56° C and 30 sec at 72° C). All primers generated amplicons that produced a single sharp peak during melting curve analysis. All PCR reactions were performed in triplicate.

Data were analyzed using relative quantitation and the comparative C_T method as described in the Applied Biosystems User Bulletin (46). Validation assays were performed by amplification of the target and reference gene, separately, using serial dilutions of an mRNA sample. Both target and reference mRNAs had equal efficiencies of amplification. The comparative C_T method calculates changes in gene expression as a

relative fold difference between an experimental and an external calibrator sample, including a correction for non-ideal amplification efficiencies (47).

Statistical analysis

Data were described as mean ± standard deviation (SD) or median and interquartile range (25% to 75%). Comparisons between group means were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey *post hoc* test. Pearson's rank correlation coefficients were calculated between variables using a two-tailed test for significance. Log_{10} transformation was used to normalize the distribution of non-Gaussian variables, and mean values were back-transformed for presentation. Categorical variables were compared using the Pearson chi-square test. The level of statistical significance was set at $p < 0.05$. The Statistical Package for the Social Sciences 16 (SPSS, Chicago, IL,) was used for the analyses.

RESULTS

Table 1 summarizes the clinical and hormonal profiles of women in the control and PCOS groups. Patients with PCOS were divided into normotensive and hypertensive subgroups according to their SBP and DBP (3). The three groups had similar BMI ($p = 0.211$) and percentage of body fat ($p = 0.057$). As expected, hirsutism score, LH, TT and FAI were higher in the PCOS group ($p < 0.001$). The analysis of hyperandrogenism revealed that TT levels and FAI were strikingly higher ($p < 0.001$), whereas SHBG levels were lower in the hypertensive PCOS group ($p = 0.025$). Although patients with hypertension and PCOS were younger than controls ($p < 0.001$), they had hyperinsulinemia, as indicated by HOMA-IR and fasting insulin ($p \leq 0.001$). Fibrinogen and WBC were similar, but hs-CRP was significantly higher in the hypertension than in the control group.

Subcutaneous CYP19 mRNA was significantly higher in the hypertensive PCOS group than in the control and normotensive PCOS groups (3.839 ± 1.010 vs. 2.891 ± 0.995 and 2.822 ± 0.761 n fold change from the calibrator sample; $p = 0.016$) (Figure 1). Nonetheless, the analysis of the estrogen-to-androgen ratio in the group of women with PCOS and normal blood pressure and in the hypertensive PCOS group showed that results in the first were similar to control group results, but the ratio in the second was lower [0.05 (0.04-0.08) vs. 0.04 (0.02-0.05) vs. 0.07 (0.06-0.17); $p < 0.001$] (Table 1). Moreover, an estrogen-to-androgen ratio ≤ 0.06 was observed in 91% of hypertensive PCOS women, vs. 37% and 61%, respectively, in the control and normotensive PCOS groups ($p = 0.011$; Figure 2).

Interestingly, CYP19 gene expression in subcutaneous fat of patient with PCOS correlated positively with SBP ($p = 0.006$) and DBP ($p = 0.009$), as shown in Figure 3. No other significant correlations were found between subcutaneous CYP19 expression and hormonal variables.

DISCUSSION

In this study, patients with PCOS were divided into normotensive or hypertensive subgroups and compared with the normotensive control group. The two PCOS subgroups had higher androgen levels and a worse metabolic profile than the control group, which confirms findings reported in previous studies conducted by our research team (14, 19, 34, 35, 41, 48) and other authors (49-53). Along with obesity and insulin resistance, hypertension is one of the major risk factors for metabolic syndrome, a known determinant of cardiovascular disease (3, 19, 24, 54-56). In addition, the presence of androgen excess, endothelial dysfunction and higher circulating inflammatory factors, as observed in the PCOS phenotype, may contribute to higher cardiovascular risk (21, 23-26, 56, 57). Our patients with hypertension and PCOS were even more hyperandrogenic and insulin resistant than patients in the normotensive groups.

We chose to measure CYP19 gene expression only in subcutaneous fat because evidence suggests that there is greater aromatase expression in subcutaneous than in visceral fat (58, 59). Another advantage of using subcutaneous fat is that the procedure for obtaining samples is less invasive than for visceral fat. Forty-seven (81%) of the women in our PCOS and control groups agreed to undergo abdominal adipose tissue biopsy, and we did not have to rely on volunteers undergoing a variety of elective surgical procedures, a limitation of previous studies.

Aromatase is the key enzyme for estrogen synthesis from androgens. Although patients in the hypertensive PCOS group had higher subcutaneous CYP19 expression, estradiol levels were similar to those found for the normotensive PCOS and control groups. In addition, the estrogen-to-androgen ratio was lower in the hypertensive PCOS group than in the control group, which strongly suggests that there are even higher androgen excess in patients with PCOS and high blood pressure. Excessive circulating androgens, found in women in the hypertensive PCOS group, may activate CYP19 mRNA transcription in adipose tissue because androgens are substrates for the aromatase enzyme. In fact, the rate of an elementary reaction is proportional to the product of the concentrations of the participating molecules. Moreover, evidence suggests that estrogens synthesized in extra gonadal sites are only partially secreted into the circulation. Rather, they exert intracrine, autocrine, or paracrine effects and, thus, act locally or in neighboring tissues (60-63), which might partially explain the absence of differences on estradiol serum levels between our study groups.

There are intricate associations with sex hormones in hypertension. Androgens are known to play an important role on cardiovascular outcomes and in the development of vascular events, which is confirmed by the fact that age-matched blood pressure is consistently higher in men than in women; however, these differences are attenuated when women reach menopause (24, 31). In addition, in women with PCOS, hypertension has been linked to androgen excess and insulin resistance (4, 5, 15-22), as observed in our study.

In addition to the proteins involved in lipid and steroidogenic metabolism, adipose tissue is currently known to secrete a large number of cytokines and growth factors, namely adipokines. These products of adipose secretory activity control various physiological and pathological processes of the body (64, 65). In fact, aromatase expression in adipose fat is stimulated primarily by class I cytokines [e.g., interleukin (IL)-6, IL-11, leukemia inhibitory factor (LIF), oncostatin-M] and tumor necrosis factor alpha produced locally in the presence of systemic glucocorticoids and secondly by prostaglandin E2 via promoter I.4 located 70 kb upstream of the CYP19 gene encoding region (27, 62, 66). Moreover, previous studies evaluated aromatase gene expression in adipose tissue specimens collected from relatively healthy middle-age man before and after elective cardiac surgery, and findings suggested that the increased postoperative adipose tissue CYP19 mRNA expression was caused by induction of the aromatase gene by factors involved in critical illness, such as the evident elevation of circulating levels of cortisol and IL6 in the study patients (67).

Conversely, studies have found some associations between estrogen-related genes and hypertension. Some of these studies have investigated the association of CYP19 gene variants and hypertension, abnormal SBP, DBP, or both, and adiposity with increased risk of myocardial infarction and coronary artery disease. Their results suggest that there are gender-specific contributions of CYP19 gene polymorphism to blood pressure variation (29, 31, 32, 68).

Although the molecular mechanisms involved in some physiological and pathological processes associated with androgen and estrogen conversion remain unclear, increased aromatase expression and activity in adipose tissue have been associated with aging and obesity for both sexes (58, 63, 69). Hyperinsulinemia due to insulin resistance plays a role in the abnormal steroidogenesis and follicle development, which worsens the ovarian dysfunction seen in PCOS (5). The formation of estradiol depends on both LH and FSH. LH stimulates production of androgens that are used in

estrogen production, while FSH directly activates the aromatase enzymatic complex to catalyze the conversion of androgen into estrogen.

In conclusion, the association between subcutaneous aromatase gene expression and blood pressure in women with PCOS suggests that androgen excess and hyperinsulinemia may play a role in the molecular mechanisms that activate the aromatase mRNA transcription in abdominal fat tissue. Further studies should assess the interaction of CYP19 gene expression and inflammatory adipokines.

ACKNOWLEDGMENTS

We thank Dr. Rinaldo de Angeli Pinto and his staff at the Plastic Surgery Service at Hospital de Clínicas de Porto Alegre, Brazil, for performing the biopsies of periumbilical adipose tissue.

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Table 1: Clinical and hormonal features of women in control and PCOS groups

	Control group (n = 27)	PCOS		p
		Normotensive (n = 20)	Hypertensive (n = 11)	
Age (years)	30.1 ± 4.5 [a]	25.5 ± 5.3 [b]	21.1 ± 6.1 [b]	**<0.001**
BMI (kg/m^2)	30 ± 5	29 ± 6	32 ± 6	0.211
Body fat (%)	26.5 ± 5.7	26.6 ± 6.3	31.6 ± 6.4	0.057
Ferriman-Gallwey score	2 (0-5) [a]	9 (8-13) [b]	14 (5-21) [b]	**0.001**
Systolic BP (mmHg)	113 ± 7 [a]	109 ± 10 [a]	134 ± 13 [b]	**<0.001**
Diastolic BP (mmHg)	72 ± 7 [a]	69 ± 9 [a]	83 ± 10 [b]	**<0.001**
Fibrinogen (mg/dL)	321 ± 50	337 ± 95	357 ± 93	0.441
hs-CRP (mg/L)	1.6 (0.7-4.0) [a]	1.9 (0.7-11.7) [ab]	5.0 (3.4-10.3) [b]	**0.046**
WBC ($10^3/\mu$L)	6.074 ± 1.639	6.986 ± 1.895	6.919 ± 0.991	0.130
Fasting insulin (uIU/mL)	9 (5-12) [a]	11 (7-13) [a]	18 (15-28) [b]	**<0.001**
HOMA-IR	2.0 (1.2-2.7) [a]	2.1 (1.5-3.2) [a]	4.2 (3.2-7.0) [b]	**0.001**
FSH (mIU/mL)	5.6 (4.4-7.0)	5.7 (4.9-6.6)	5.6 (5.2-6.4)	0.936
LH (mIU/mL)	5.4 (3.8-7.9) [a]	10.6 (6.4-14.9) [b]	14.7 (11.1-16.6) [b]	**<0.001**
SHBG (nmol/L)	43.6 (29.6-51.6) [a]	41.4 (23.3-47.9) [ab]	22.1 (12.6-57.9) [b]	**0.025**
17OHP (ng/mL)	0.7 (0.4-1.1) [a]	0.9 (0.6-1.2) [ab]	1.4 (1.2-2.0) [b]	**0.008**
DHEAS (μg/dL)	128.8 (88.4-176.2)	185.1 (94.2-242.5)	250.1 (151.3-268.8)	0.083
Total testosterone (ng/mL)	0.6 (0.5-0.7) [a]	0.9 (0.8-1.2) [b]	1.5 (1.3-2.0) [c]	**<0.001**
FAI	4.5 (3.9-6.9) [a]	7.9 (5.4-13.2) [b]	19.8 (11.8-41.9) [c]	**<0.001**
Estradiol (pg/mL)	45.7 (33.0-97.0)	53.7 (39.7-69.0)	55.0 (39.7-57.8)	0.889
Estrogen to androgen ratio	0.07 (0.06-0.17) [a]	0.05 (0.04-0.08) [ab]	0.04 (0.02-0.05) [b]	**<0.001**

Values are expressed as mean ± SD or median and interquartile range (25% to 75%). One-way ANOVA + Tukey post hoc test. 17OHP: 17-hydroxyprogesterone; BP: blood pressure; BMI: body mass index; DHEAS: dehydroepiandrosterone sulfate; FAI: free androgen index; FSH: follicle stimulating hormone; HOMA-IR: homeostasis model assessment; hs-CRP: highly sensitive C-reactive protein; LH: luteinizing hormone; PCOS: polycystic ovary syndrome; SHBG: sex hormone binding globulin; WBC: white blood cells.

Figure 1:

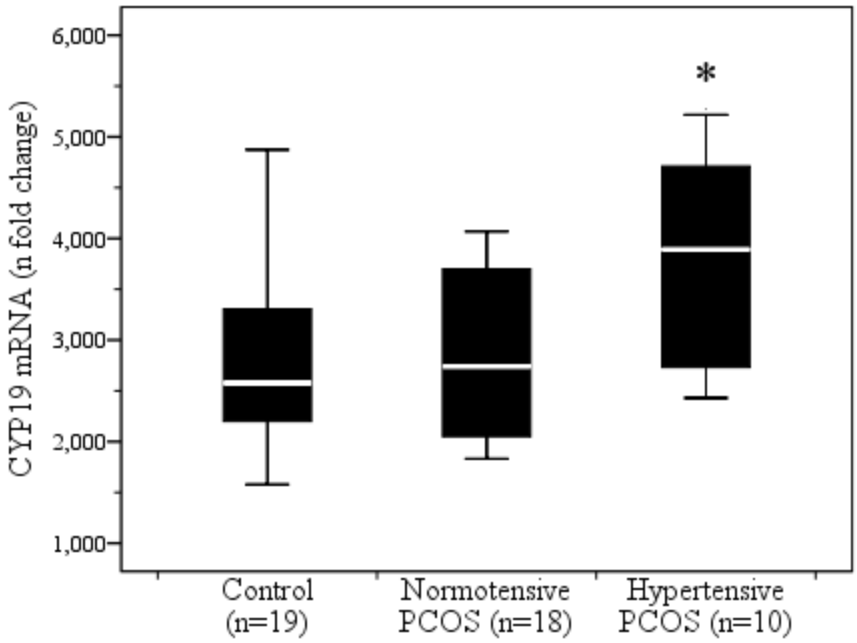

6,000
5,000
4,000
3,000
2,000
1,000
CYP19 mRNA (n fold change)
*
Control (n=19)
Normotensive PCOS (n=18)
Hypertensive PCOS (n=10)

Figure 2:

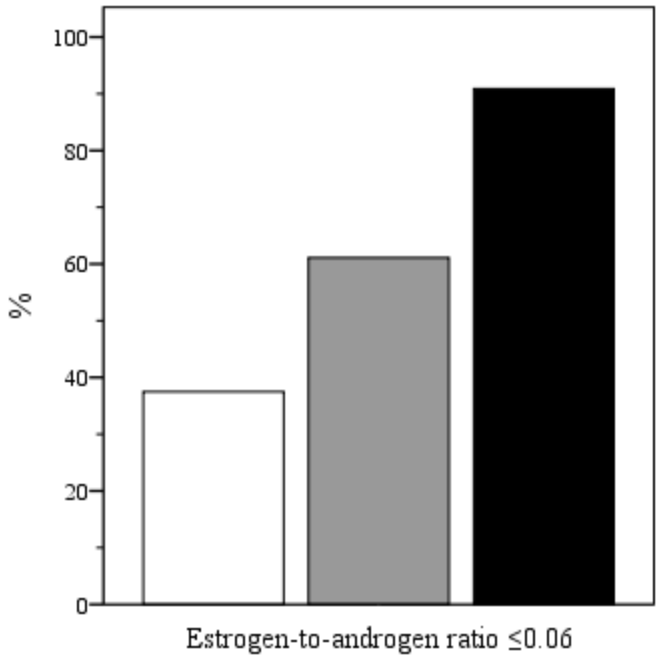
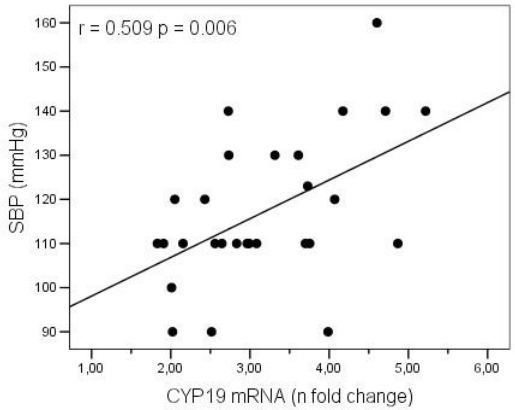


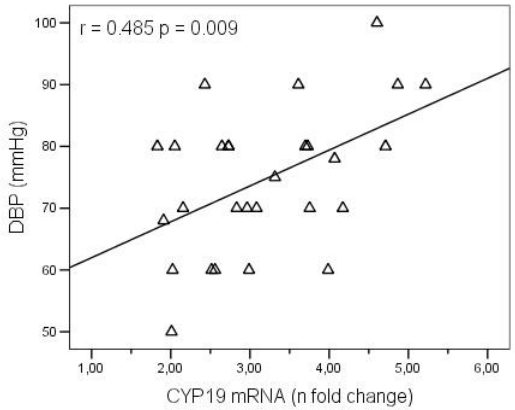
Figure 3:

PCOS

A

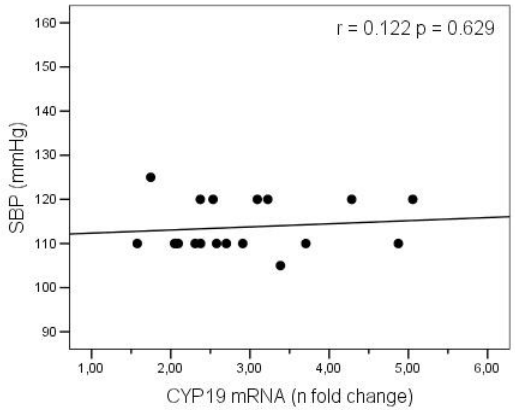


B



CONTROL

C



D

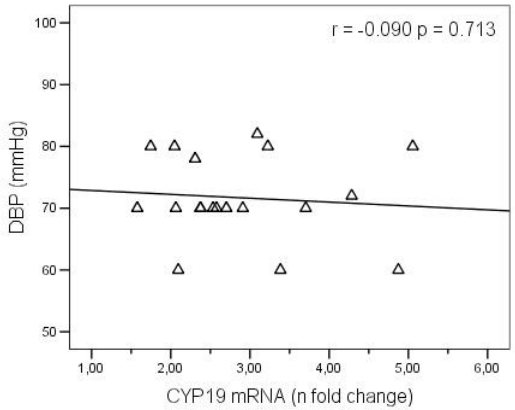


FIGURE LEGENDS

Figure 1: CYP19 gene expression in subcutaneous adipose tissue of women in the control group and in polycystic ovary syndrome (PCOS) patients divided according to the presence of hypertension (NCEP/ATP III). Messenger ribonucleic acid (mRNA) was expressed as n fold change difference from the calibrator sample ($\Delta\Delta C_T$ method). Number of biopsies analyzed per group is within parentheses. Values are expressed as median and interquartile range (25% to 75%). *p = 0.016 in comparison with control and normotensive PCOS groups by one-way ANOVA + Tukey *post hoc* test.

Figure 2: Prevalence of estrogen-to-androgen ratio ≤ 0.06 in control [□], normotensive polycystic ovary syndrome (PCOS) [■], and hypertensive PCOS [■] groups. The cut-off point expresses the median estrogen-to-androgen ratio in control and PCOS (with or without hypertension) women. p = 0.011 by Pearson chi-square test.

Figure 3: Correlation between CYP19 gene expression in subcutaneous adipose tissue and **(A and C)** systolic blood pressure (SBP) in polycystic ovary syndrome (PCOS) and control groups, and **(B and D)** diastolic blood pressure (DBP) in PCOS and control groups. Messenger ribonucleic acid (mRNA) was expressed as n fold change difference from the calibrator sample ($\Delta\Delta C_T$ method). Pearson's rank correlation coefficients.

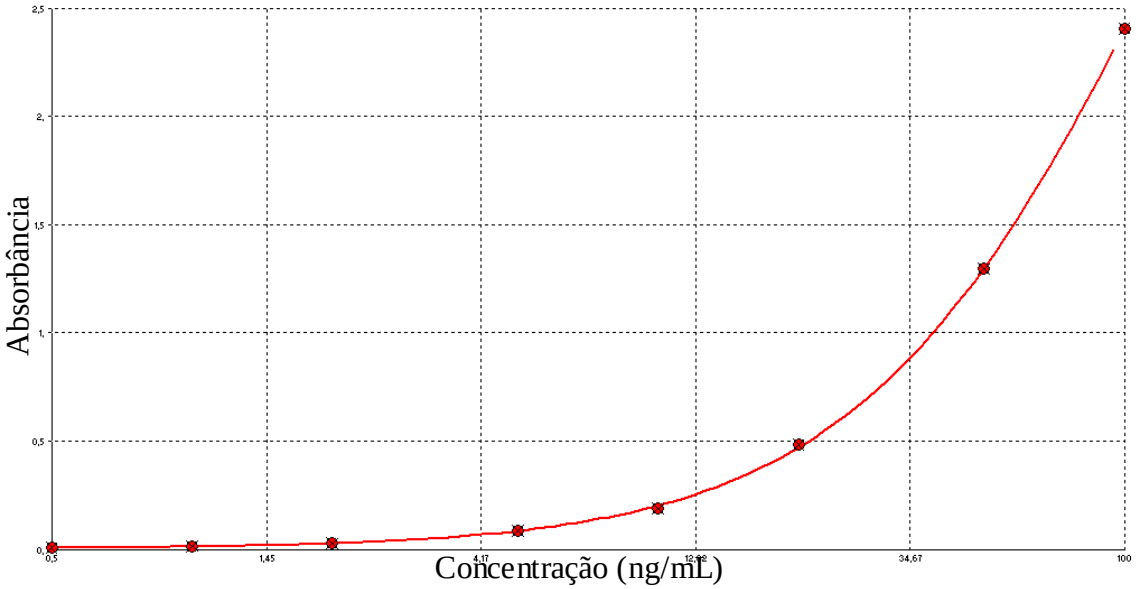
ANEXOS

LEPTINA Plate-ID: 20090318-Leptina

Standards

Response Formula	nominal	adjusted	OD
S1	0,50	0,55	0,007
S2	1,00	1,24	0,016
S3	2,00	2,01	0,028
S4	5,00	5,06	0,087
S5	10,00	9,41	0,188
S6	20,00	20,43	0,483
S7	50,00	49,87	1,296
S8	100,00	>100,00	2,406

4-Paramter Fit
A = 0,002149
B = 1,294866
C = 131,009430
D = 5,814837
Axis (X/Y): lin/log



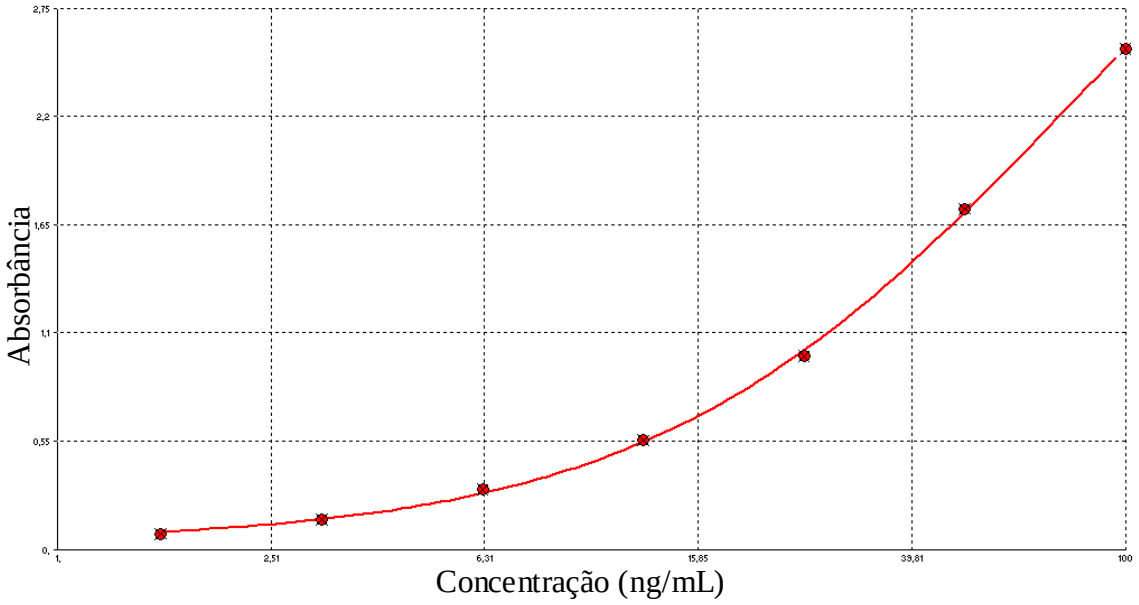
Anexo 1: Curva de calibração representativa dos ensaios para determinação de leptina sérica (Human Leptin ELISA kit, LINCO Research, Missouri, USA). Espectrofotômetro Anthos Zenyth 200rt , software ADAP Prisma versão 1.6.

Adiponectina Plate-ID: 20090211-Adipo

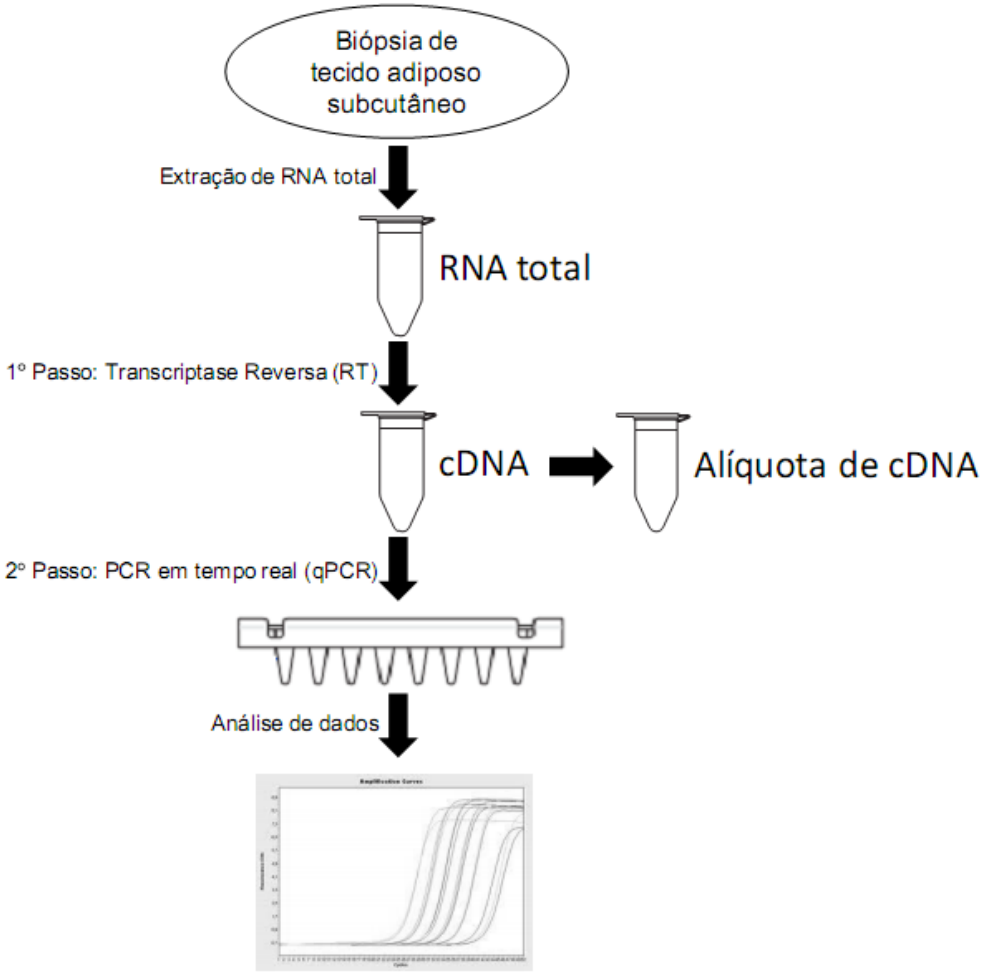
Standards

Response Formula	nominal	adjusted	OD
S1	1,56	<1,56	0,080
S2	3,13	3,07	0,151
S3	6,25	6,66	0,303
S4	12,50	12,73	0,555
S5	25,00	24,17	0,982
S6	50,00	50,78	1,731
S7	100,00	99,64	2,544

4-Paramter Fit
A = 0,032984
B = 1,114489
C = 76,723969
D = 4,420490
Axis (X/Y): lin/log



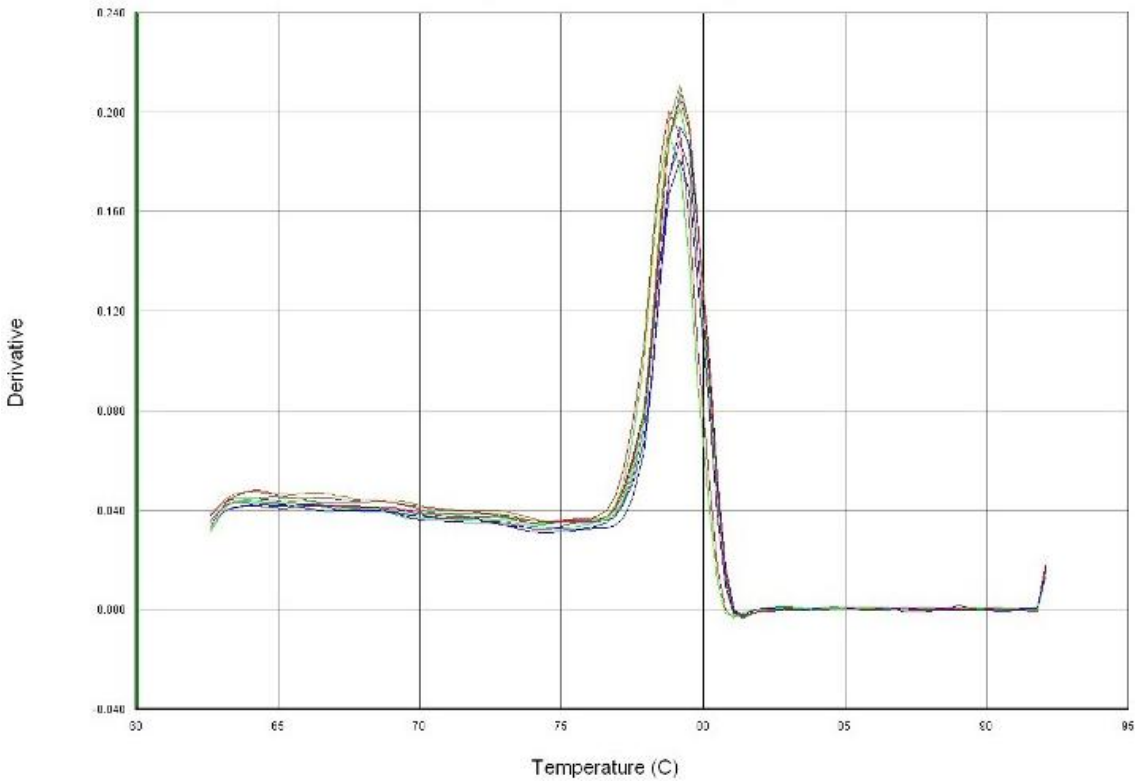
Anexo 2: Curva de calibração representativa dos ensaios para determinação de adiponectina sérica (Human Adiponectin ELISA kit, LINCO Research, Missouri, USA). Espectrofotômetro Anthos Zenyth 200rt , software ADAP Prisma versão 1.6.



Anexo 3: Fluxograma de trabalho: expressão gênica dos fragmentos dos genes de interesse pela técnica de transcriptase reversa seguida da reação em cadeia da polimerase com quantificação em tempo real (RT-qPCR).

Detector: Leptina

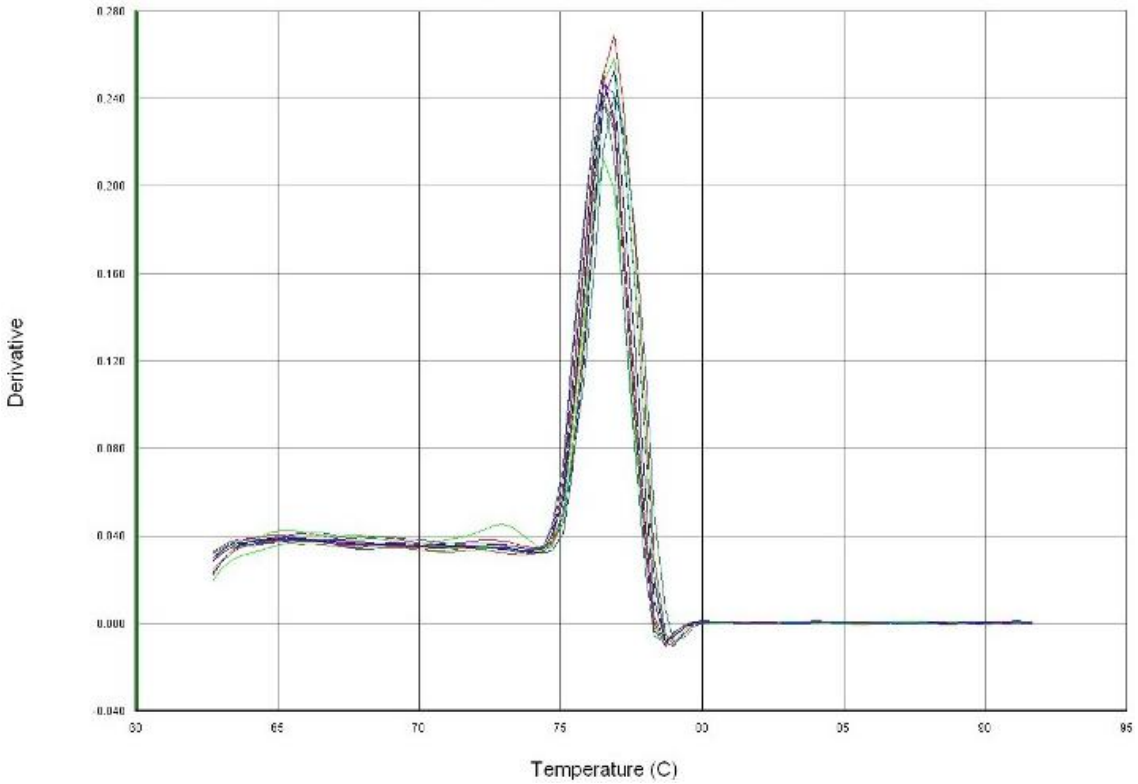
Documento: 050710 (Standard Curve)



Anexo 4: Curva de dissociação representativa dos ensaios de expressão gênica em tecido adiposo subcutâneo para leptina. Termociclador 7500 Fast Real-Time PCR System/7500 Fast System Sequence Detection 1.4 Software (Applied Biosystems, Foster City, USA).

Detector: Adiponectina

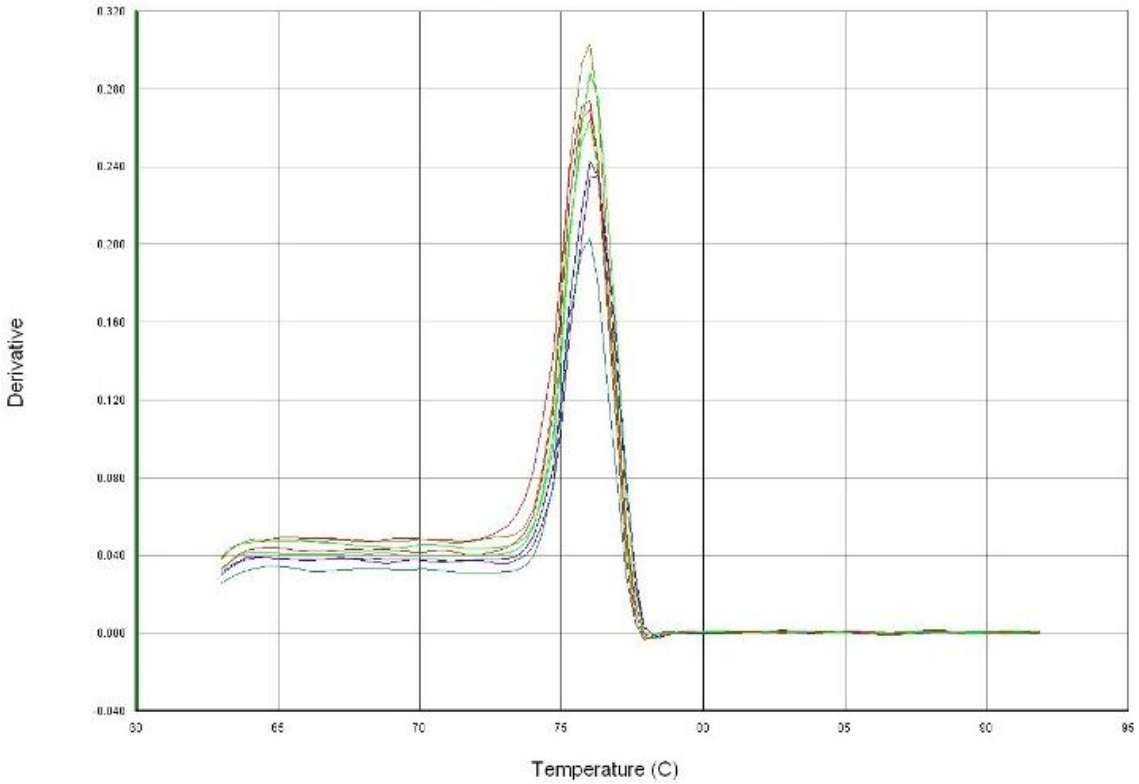
Documento: 100709 (Standard Curve)



Anexo 5: Curva de dissociação representativa dos ensaios de expressão gênica em tecido adiposo subcutâneo para adiponectina. Termociclador 7500 Fast Real-Time PCR System/7500 Fast System Sequence Detection 1.4 Software (Applied Biosystems, Foster City, USA).

Detector: Aromatase

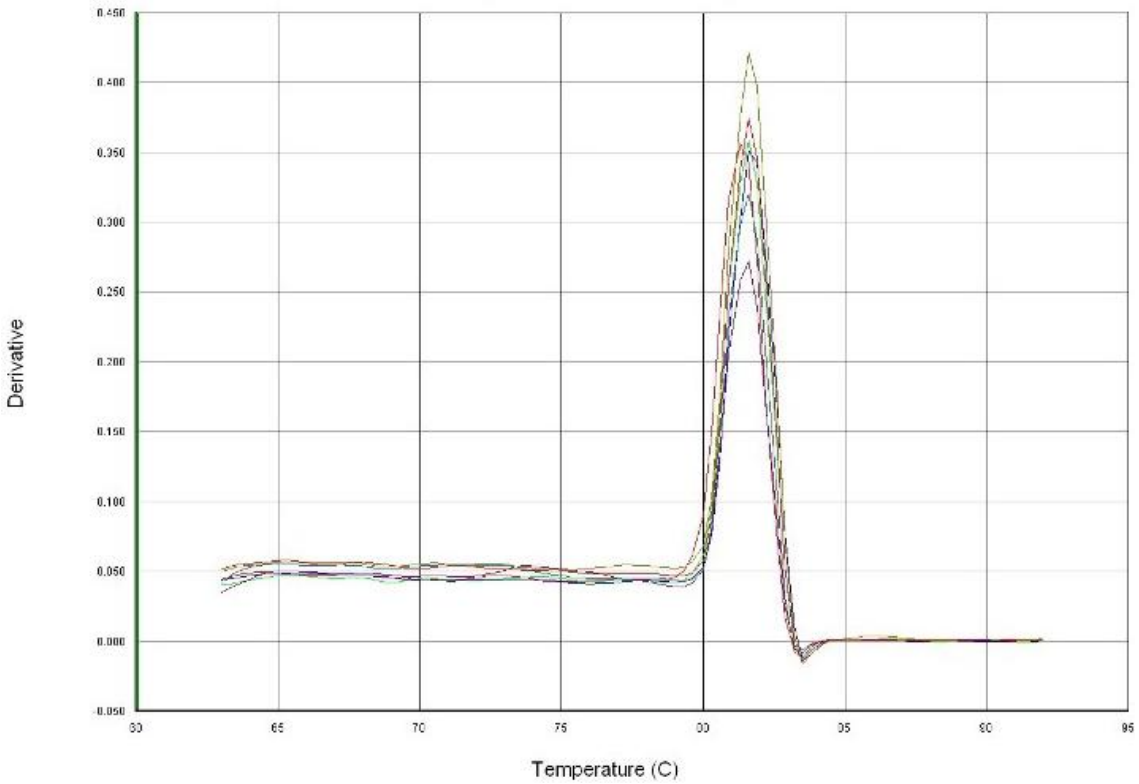
Documento: 060310 (Standard Curve)



Anexo 6: Curva de dissociação representativa dos ensaios de expressão gênica em tecido adiposo subcutâneo para CYP19 (aromatase). Termociclador 7500 Fast Real-Time PCR System/7500 Fast System Sequence Detection 1.4 Software (Applied Biosystems, Foster City, USA).

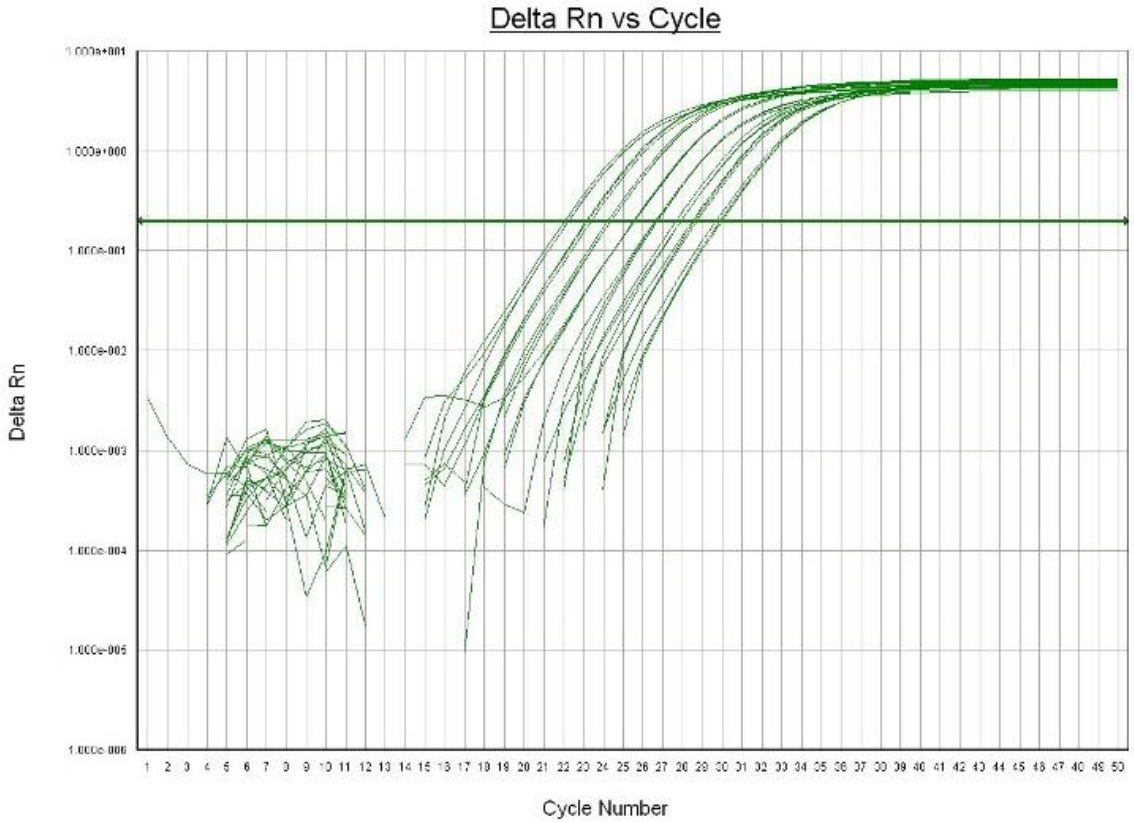
Detector: GAPDH

Documento: 160810 (Standard Curve)



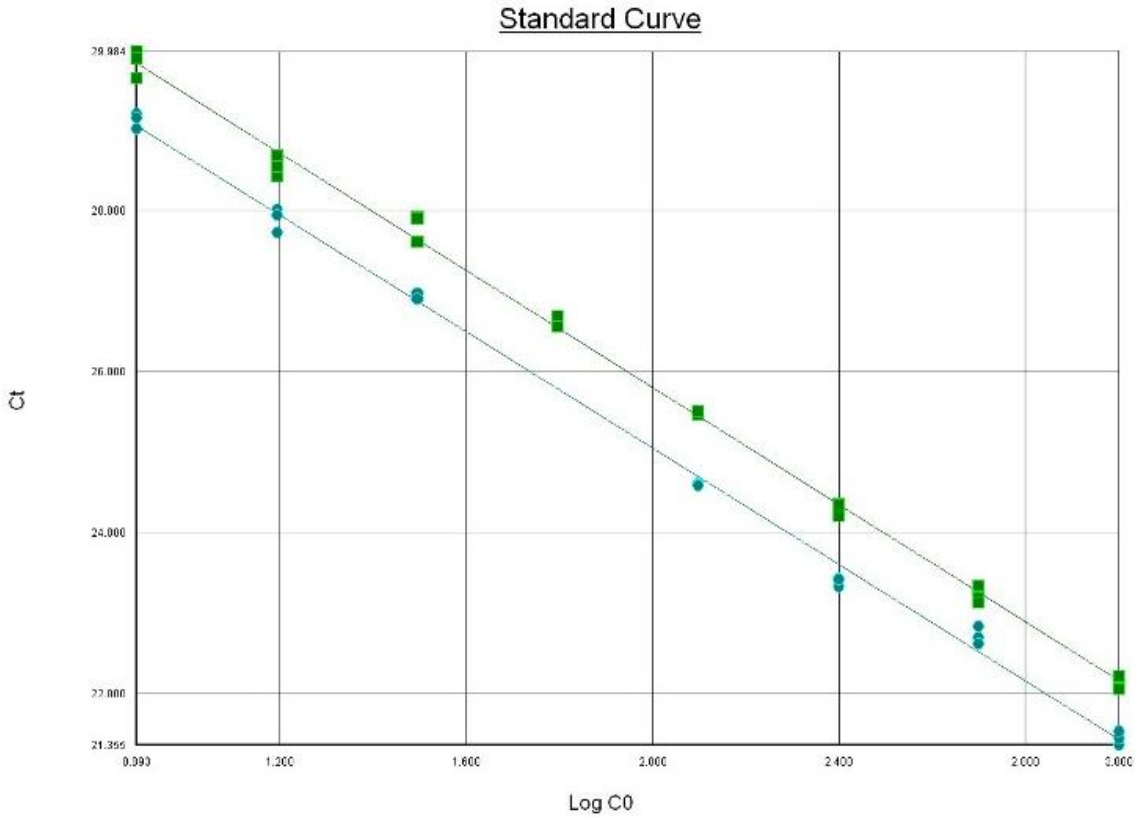
Anexo 7: Curva de dissociação representativa dos ensaios de expressão gênica em tecido adiposo subcutâneo para gliceraldeído 3 fosfato dehidrogenase (GAPDH). Termociclador 7500 Fast Real-Time PCR System/7500 Fast System Sequence Detection 1.4 Software (Applied Biosystems, Foster City, USA).

Documento: Curva 070810 (Standard Curve)



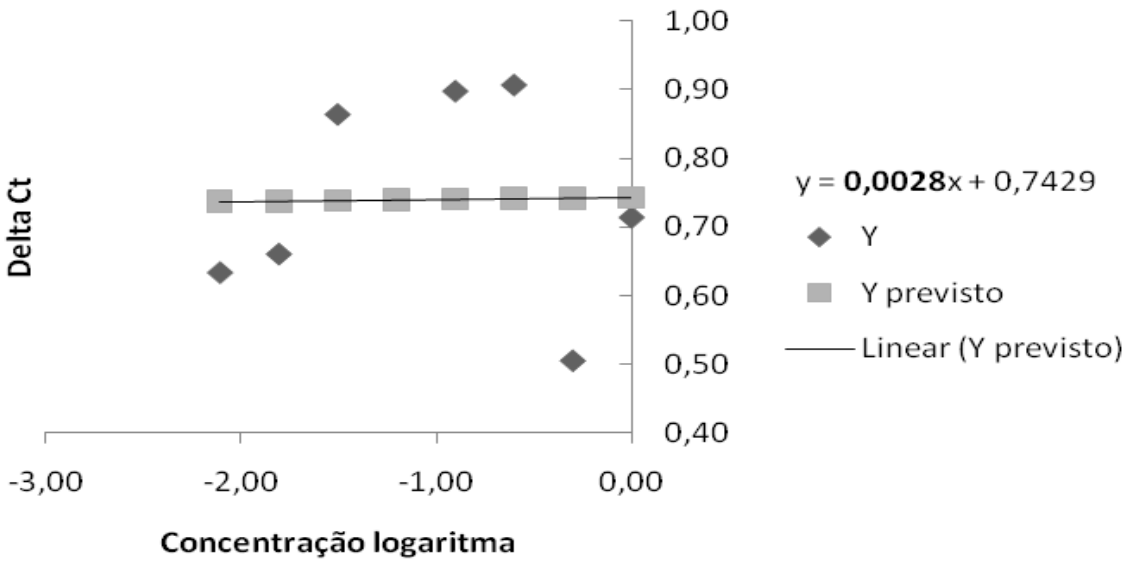
Anexo 8: Curva de padronização relativa representativa dos ensaios de validação para expressão gênica em tecido adiposo subcutâneo: diluição seriada da amostra padrão na ordem de 1/128 apresentada como ΔRn vs. número de ciclos da reação. Termociclador 7500 Fast Real-Time PCR System/7500 Fast System Sequence Detection 1.4 Software (Applied Biosystems, Foster City, USA).

Documento: Curva 070810 (Standard Curve)



Anexo 9: Curva de padronização relativa representativa dos ensaios de validação para expressão gênica em tecido adiposo subcutâneo: diluição seriada da amostra padrão na ordem de 1/128 para o gene de interesse e controle endógeno, apresentada como C_T vs. concentração logaritma das diluições seriadas. Termociclador 7500 Fast Real-Time PCR System/7500 Fast System Sequence Detection 1.4 Software (Applied Biosystems, Foster City, USA).

Documento: Curva 070810 (Standard Curve)



Anexo 10: Equação linear da reta representativa do experimento de validação para expressão gênica em tecido adiposo subcutâneo: os valores do ΔC_T foram plotados em relação à concentração logaritma de cada ponto de diluição da curva de padronização relativa. [Relative quantitation of gene expression experimental design and analysis: relative standard curve method and comparative ct method (ΔΔct). Guide to performing relative quantitation of gene expression using real-time quantitative pcr. Foster City, California, USA: Apllied Byosystems, 2004:34-49].

L461n Lecke, Sheila Bünecker

Níveis séricos e expressão gênica de leptina, adiponectina e aromatase em tecido adiposo de mulheres com a síndrome dos ovários policísticos / Sheila Bünecker Lecke ; orient. Poli Mara Spritzer ; co-orient. Débora Martinho Morsch. – 2010.

99 f. : il.

Tese (doutorado) – Universidade Federal Rio Grande do Sul. Faculdade de Medicina. Programa de Pós-Graduação em Ciências Médicas: Endocrinologia. Porto Alegre, BR-RS, 2010.

1. Síndrome do ovário policístico 2. Tecido adiposo 3. Expressão gênica 4. Leptina 5. Adiponectina 6. Aromatase I. Spritzer, Poli Mara II. Morsch, Débora Martinho III. Título.

NLM: WP 320

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