1. Introduction

1.1 Interstitial cells of Leydig in the testes

Interstitial cells of Leydig secrete testosterone and are located in the interstices between the seminiferous tubules in the testes. The mammalian Leydig cell is a polyhedral epithelioid cell with a single eccentrically located ovoid nucleus. The nucleus contains one to three prominent nucleoli and large amounts of dark-staining peripheral heterochromatin. The acidophilic cytoplasm usually contains numerous membrane-bound lipid droplets and large amounts of smooth endoplasmic reticulum (SER). Besides the obvious abundance of SER with scattered patches of rough endoplasmic reticulum, several mitochondria are also prominent within the cytoplasm. Frequently, lipofuscin pigment and rod-shaped crystal-like structures 3 to 20 micrometres in diameter (Reinke's crystals) are found. These inclusions have no known function. No other interstitial cell within the testes has a nucleus or cytoplasm with these characteristics, making identification relatively easy (fig.1).

Franz Leydig first described Leydig cells in the 1850s. He observed cells in the testis with dense granules which were later shown to be lipid droplets. This morphological feature predicted the function of Leydig cells. They convert cholesterol to testosterone, and the lipid droplets are vesicles filled with cholesterol esters. These lipid droplets provide cholesterol for testosterone production. Despite the observation of Leydig cells in the testis, it wasn’t until the late 1950s and early 1960s that is was shown unequivocally that they are the cells that produce testosterone. Studying the testis they were unraveled the steps of steroid hormone biosynthesis. Early studies determined that cholesterol is converted to pregnenolone within the mitochondria and then pregnenolone is converted in the smooth endoplasmic reticulum to the final

steroid products. These studies also showed that the first hormonally regulated step in steroid synthesis is the conversion of cholesterol to pregnenolone (*Guyton et al. 2006).*

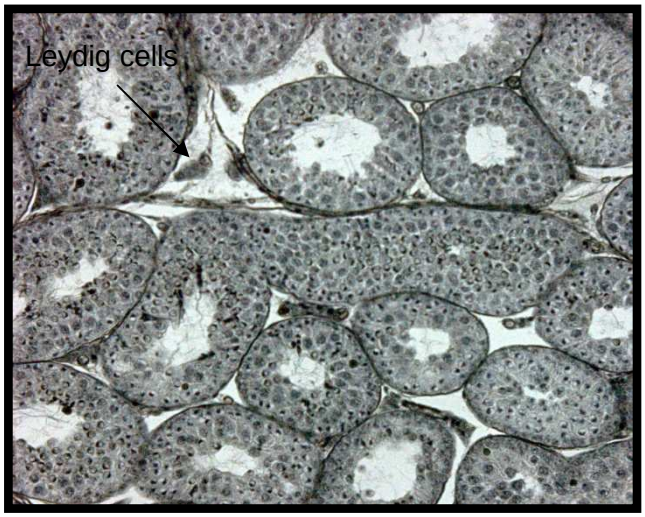

Fig.1 *Interstitial cells of Leydig in the testes.*

1.2 Biosynthesis of testosterone

During the 1970s the pathway of testosterone production was elucidated and shown to comprise many of the same steps that result in adrenal steroid synthesis, and performed by many of the same enzymes. The first enzyme in the pathway is a cholesterol side-chain cleavage enzyme referred to as P450scc. P450scc resides on the inner face of the inner mitochondrial membrane. The next enzyme in the pathway is 3β-hydroxysteroid dehydrogenase which is referred to as 3β-HSD. 3β-HSD converts pregnenolone to progesterone, the first biologically active steroid hormone in the pathway. Progesterone is then converted to androstenedione by the action of 17α-hydroxylase/c17-20 lyase enzyme, referred to as P450c17. Androstenedione is the immediate precursor of testosterone.The final step in the biosynthesis, conversion of androstenedione to testosterone is carried out by the enzyme 17β-hydroxysteroid dehydrogenase, referred to as 17β-HSD (fig 2).

The same basic pathway is utilized for testosterone production in all mammals, though the order in which the enzymes act may differ slightly. Another feature of steroid hormone synthesis common to the adrenals and gonads is the control by pituitary tropic hormones. Adrenal glucocorticoid hormones such as cortisol are under the control of adrenocorticotrophic hormone or ACTH. Ovarian and testicular steroid hormone production is under the control of the gonadotropins, Luteinizing hormone and follicle stimulating hormone, or LH and FSH, respectively. Testosterone production by Leydig cells is under the control of LH.

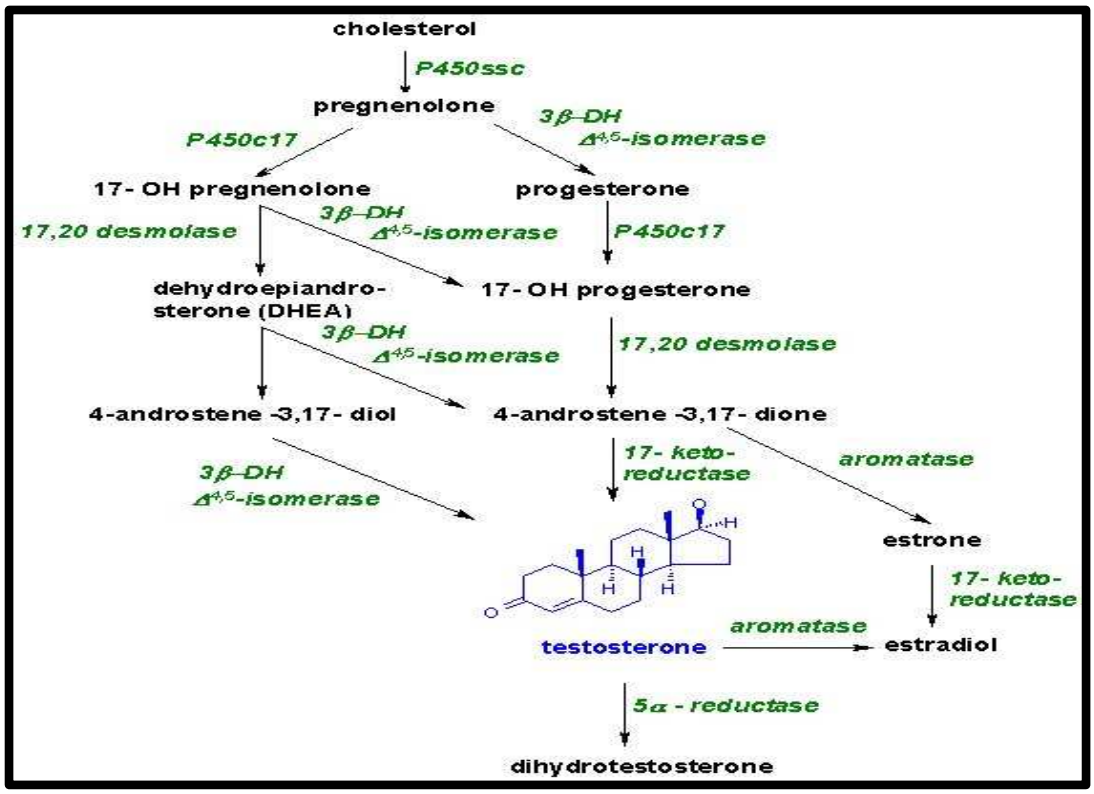

Fig. 2 *Biosynthetic pathway of testicular testosterone synthesis*

LH is secreted by the anterior pituitary gland and travels via the blood stream to the testes where it binds to receptors on the surface of the Leydig cells and stimulates testosterone production. LH is what is known as a glycoprotein hormone and it can't enter the cell. Instead it binds to receptors on the outside surface of the cell and activates an intracellular second messenger system inside the cell to provoke cellular responses. CyclicAMP, referred to as cAMP, is LH's second messenger in Leydig cells. LH actions in Leydig cells are signaled via the production of cAMP which activates the enzyme protein kinase A which results in the phosphorilation of hormone sensitive lipase and perilipin involving in the hidrolysis and mobilization of cholesteryl esters stored in lipid droplets. The resulting free cholesterol (precursor for testosterone synthesis) is then transported from the outer to the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR), where it is metabolized to pregnenolone by P450scc *(Hu et al. 2010).* It is believed that once

converted to pregnenolone, this more hydrophilic steroid is free to diffuse out of the mitochondria into the microsomes for further conversion to specific steroids dependent upon the battery of steroidogenic enzymes *(Stocco et al. 1997*) (fig.3).

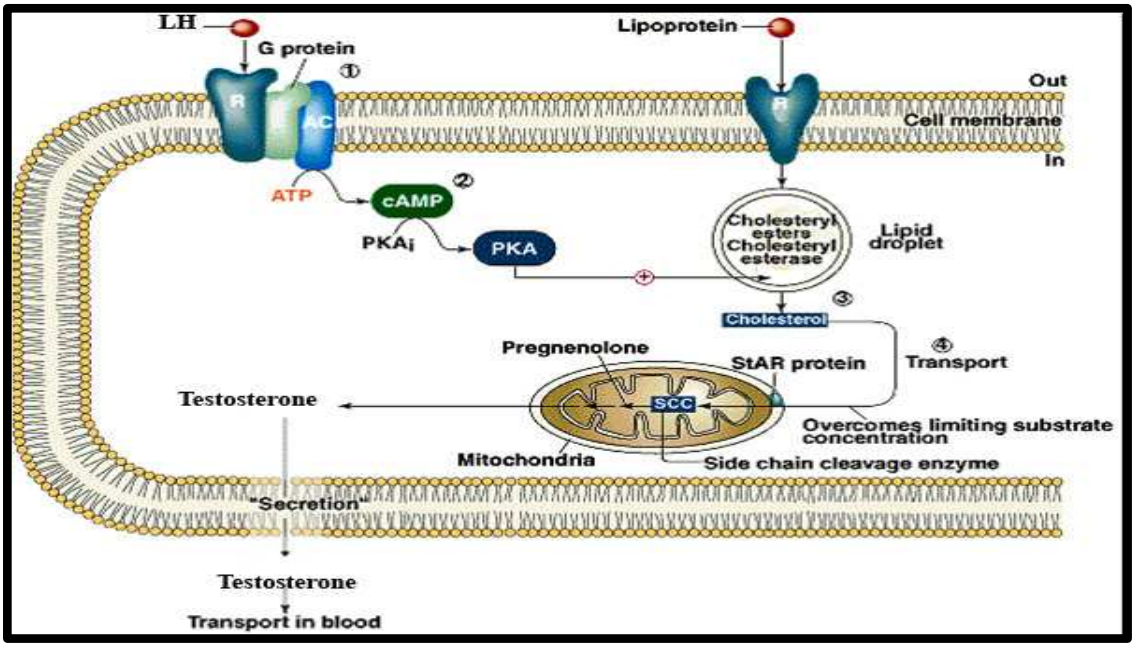

Fig.3 *Model showing testosterone synthesis in Leydig cells*

After secretion by the Leydig cells about 97% of the testosterone becomes either loosely bound with plasma albumin or more tighly bound with a beta globulin called sex hormone –binding globulin and circulates in the blood in these states for 30 minutes to several hours. By that time, the testosterone either is transferred to the tissues or is degraded into inactive products that are subsequently excreted. Muche of the testosterone that becomes fixed to the tissue is converted within the tissue cells to dihydrotestosterone. The testosterone that does not become fixed to the tissues is rapidly converted, mainly by the liver, into androsterone and dehydroepiandrosterone and simultaneously coniugated as either glucoronides or sulfates. These are excreted either into the gut by way of the liver bile or into the urine through the kidneys *(Guyton et al. 2006).*

1.3 Pathways regulating steroidogenesis

However, whereas the cAMP/PKA pathway is undoubtedly the major signaling cascade regulating steroidogenesis, many recent studies have indicated that additional pathways are involved in this process as well. A large body of evidence indicates that regulation of steroidogenesis can also be modulated through signal transduction pathways not involving cAMP. Indeed, several factors that do not require cAMP and/or protein synthesis have been demonstrated to potently stimulate steroidogenesis. These include growth factors, macrophage-derived factors, steroidogenic-inducing protein (SIP), chloride ions, and calcium (Ca^{2+}) messenger systems. It should be noted, however, that regardless of the stimulant, the cAMP-independent induction of steroidogenesis is quite modest when compared with the cAMP/PKA response, usually being less than 1% of that seen with the cAMP/PKA-dependent pathway. Even though the magnitude of response mediated by these factors on steroidogenesis is small, many of them are capable of potentiating the steroidogenic responsiveness of gonadal cells to gonadotropins or cAMP analogs, and, by doing so, they play important roles in regulating various testicular/ovarian functions. In addition, an overwhelming amount of data indicates that the testis produces a variety of regulatory molecules and that a local control system exists within this organ. In addition to the role of the cAMP signal transduction pathway, studies over the past three decades have demonstrated a critical role for AA-mediated signal transduction in trophic hormone-stimulated steroid biosynthesis. It has been reported that trophic hormone stimulation not only induces cAMP formation, but also results in the release of AA from intracellular stores. AA release occurs within 1 min of LH stimulation and is dependent on hormone-receptor interaction and the concentration of LH/hCG binding sites on the cell surface. Recent studies suggested

that this hormone-receptor interaction resulted in the activation of G proteins followed by the activation of phospholipase A2 (PLA2), which in turn catalyzed the release of AA from phospholipids. In addition to its direct effect on PLA2 activity, G protein activation also induced AA release through its ability to increase intracellular cAMP. In addition to the release of intracellular AA through the activation of PLA2, at least one additional AA-releasing pathway in steroidogenic cells has been demonstrated. AA release is critical for trophic hormone-stimulated steroidogenesis and StAR expression. Abayasekara et al. reported that inhibition of AA release from phospholipids reduced LH (100 ng/ml) and stimulated testosterone production approximately 80% by 2 h in rat testicular Leydig cells, without affecting intracellular levels of cAMP. They also determined that AA acts at the rate-limiting step of steroidogenesis, the transfer of the substrate cholesterol to the inner mitochondrial membrane. The mechanism of AA action in regulating steroidogenesis has been further explored. After AA is released, it is metabolized mainly through one of three enzymatic pathways, the cyclooxygenase (COX), the lipoxygenase, or the epoxygenase. It was reported that inhibition of either lipoxygenase or epoxygenase activity inhibited StAR protein expression and steroid synthesis *(Stocco et al.2005*). It was investigated that inhibition of COX activity dramatically increased the sensitivity of steroidogenesis to cAMP stimulation *(Wang et al. 2003).*

1.4 Testosterone and exercise

Testosterone (17β-hydroxy-4-androstene-3-one) is a potent anabolic hormone that stimulates muscle protein synthesis and intramuscular amino acid uptake. Testosterone is important for the development and maintenance of muscle mass in males. When circulating testosterone is suppressed, as with long-term pharmacological androgen deprivation therapy, male subjects experience a loss of muscle mass and muscle strength. As we already know a hormone receptor complex must be present for the action of the testosterone to occur. These complexes may occur in the cytosol and then translocate to the nucleus, or they may occur directly in the nucleus itself. Regardless of the location, activation of the receptor hormone-complex will increase mRNA and DNA synthesis. This increase in action will lead to increased protein accretion via increased intramuscular amino acid uptake.

Testosterone also possesses anti-catabolic properties within skeletal muscle. Inhibition of muscle glycogen breakdown and displacement of glucocorticoid via attachment to its receptor are two powerful mechanisms of skeletal muscle protein retention. Cortisol's signal is typically related to the loss of glycogen stores in the muscle and the need for glucose. Oftentimes, cortisol increases may be related to other stress factors beyond carbohydrate metabolism and may affect protein breakdown. The response of testosterone to offset this effect on protein metabolism can be vital for maintaining muscle site and function *(Loebel et al. 1998)*. The importance of testosterone for muscle adaptations to resistance exercise training was recently highlighted by the finding that suppression of circulating testosterone concentrations prevented resistance training-induced hypertrophy in young, healthy men *(Vingren et al. 2008).* It was investigated that androgens increased in response to exercise. From the perspective of an athlete, an increase in anabolic-androgenic

hormones can improve performance by decreasing body fat and increasing lean body mass and muscular strength *(Tremblay et al. 2004).* Others studies have shown that treadmill running *(Aizawa et al. 2007)* can increase muscle testosterone content acutely after exercise and that treadmill run. Considering the anabolic potency of testosterone it appears unlikely that endurance exercise favours stimulations of muscle testosterone production. Although endurance exercise training significantly increases muscle protein synthesis, this increase does not lead to muscle hypertrophy because net protein balance is not improved. Several studies have shown that resistance exercise induces an increase in myonuclei per fiber. A close relationship between myonuclear number and muscle fiber cytoplasmic volume has been reported, and this suggests a tight regulation of the quantity of genetic machinery based on the protein requirements of a muscle fiber. Since endurance training is not associated with muscle hypertrophy, it is unlikely to result in an increase in myonuclei. One could speculate that the increase in muscle testosterone found with endurance exercise in some studies is due to an accumulation because of reduced utilization and not due to increased production. It is possible that the differences among findings are due to the different training levels of the participants of the present study and the animals used in the different investigations *(Vingreen et al. 2008).* The purpose of the study of Tremblay et al. *(Tremblay et al. 2004)* was to determine the acute anabolic and catabolic hormone response to endurance and resistance exercise bouts of equal volume in subjects with differing training status *(Tremblay et al. 2004).* They observed that endurance-trained subjects displayed less pronounced changes in hormone concentrations in response to exercise than resistance-trained subjects. Endurance-trained men tend to have lower levels of testosterone compared with sedentary controls whereas resistance-trained subjects

have been shown to have higher basal testosterone levels. The main signalling peptide of testosterone, LH, may also be altered by training, with significantly elevated levels reported in endurance-trained men . Other research has focused on the ratio of testosterone to cortisol, which has been reported to both increase and decrease during resistance training. Testosterone concentrations have been shown to increase after an acute bout of resistance or endurance exercise *(Jensen et al. 1991).* In response to prolonged endurance exercise (e.g., a marathon), testosterone levels will typically decline. Others have reported no change in testosterone after resistance exercise. Jensen et al. *(Jensen et al. 1991)* found that testosterone increased significantly in men after both resistance and endurance exercise and returned to the resting level within 2 h. The magnitude and pattern of the change in testosterone were almost identical between the endurance and resistance sessions. This is in contrast to the present results where the testosterone response to resistance exercise was greater than the response to the endurance exercise session. This suggests that exercise intensity may be more predictive of the testosterone response to exercise than total energy expenditure, because Jensen et al. attempted to equate the two exercise sessions on duration and intensity *(Jensen et al. 1991).* In many cases, conflicting results can be attributed to differences in the mode or volume of exercise or in the training status of the subjects. The importance of testosterone for muscle adaptations to resistance exercise training was recently highlighted by the finding that suppression of circulating testosterone concentrations prevented resistance training-induced hypertrophy in young, healthy men (*Vingren et al. 2008*). It was investigated that androgens increased in response to exercise. From the perspective of an athlete, an increase in anabolic-androgenic hormones can improve performance by decreasing body fat and increasing lean body mass and muscular strength (*Tremblay*

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1.5 The perilipin family

Perilipins are a family of unique proteins intimately associated with the limiting surface of neutral lipid storage droplets in adipocytes and in steroidogenic cells *(Servetnick et al. 1995; Brasaemle et al. 2007).* Lipid droplets are intracellular storehouses of lipid esters. The majority of eukaryotic cells synthesize neutral lipids and package them into cytosolic lipid droplets. In vertebrates, triacylglycerol-rich lipid droplets of adipocytes provide a major energy storage depot for the body, whereas cholesteryl ester-rich droplets of many other cells provide building materials for local membrane synthesis and repair *(Dalen et al. 2007).* These lipid droplets are coated with one or more of five members of the perilipin family of proteins: adipophilin, TIP47, OXPAT/MLDP, S3-12, perilipin *(Servetnick et al. 1995).* Members of this family share varying levels of sequence similarity, lipid droplet association, and functions in stabilizing lipid droplets. The most highly studied member of the family, perilipin, is the most abundant protein on the surfaces of adipocyte lipid droplets, and the major substrate for cAMP-dependent protein kinase [protein kinase A (PKA)] in lipolytically stimulated adipocytes. Perilipin serves important functions in the regulation of basal and hormonally stimulated lipolysis. Under basal conditions, perilipin restricts the access of cytosolic lipases to lipid droplets and thus promotes triacylglycerol storage. In times of energy deficit, perilipin is phosphorylated by PKA and facilitates maximal lipolysis by HSL and adipose triglyceride lipase. A model is discussed whereby perilipin serves as a dynamic scaffold to coordinate the access of enzymes to the lipid droplet in a manner that is responsive to the metabolic status of the adipocyte.

In mice and humans, a single perilipin gene gives rise to at least three protein isoforms (perilipin A, B and C) that share a common N-terminal region and differ in their C-terminal tails.

The shared region encompasses the PAT domain, three of the 6 recognized protein kinase A (PKA) sites, a stretch of 16 aspartate and glutamate residues (the acidic loop region), and two of three hydrophobic regions that target the protein to lipid droplets. These regions have been hypothesized to act as hydrophobic fingers that dip into the non-polar core of the lipid droplet . A fourth isoform, perilipin D, has been predicted based on isolation of cDNAs that contain an in-frame stop codon within an unspliced intron; to our knowledge no protein that corresponds to the predicted perilipin D has been identified and reported. Perilipin A is the most abundant isoform , and the focus of almost all functional studies on perilipins. Perilipin B is expressed on adipocyte and steroidogenic cells lipid droplet, while perilipin C is expressed only in steoidogenic cells (fig.4).

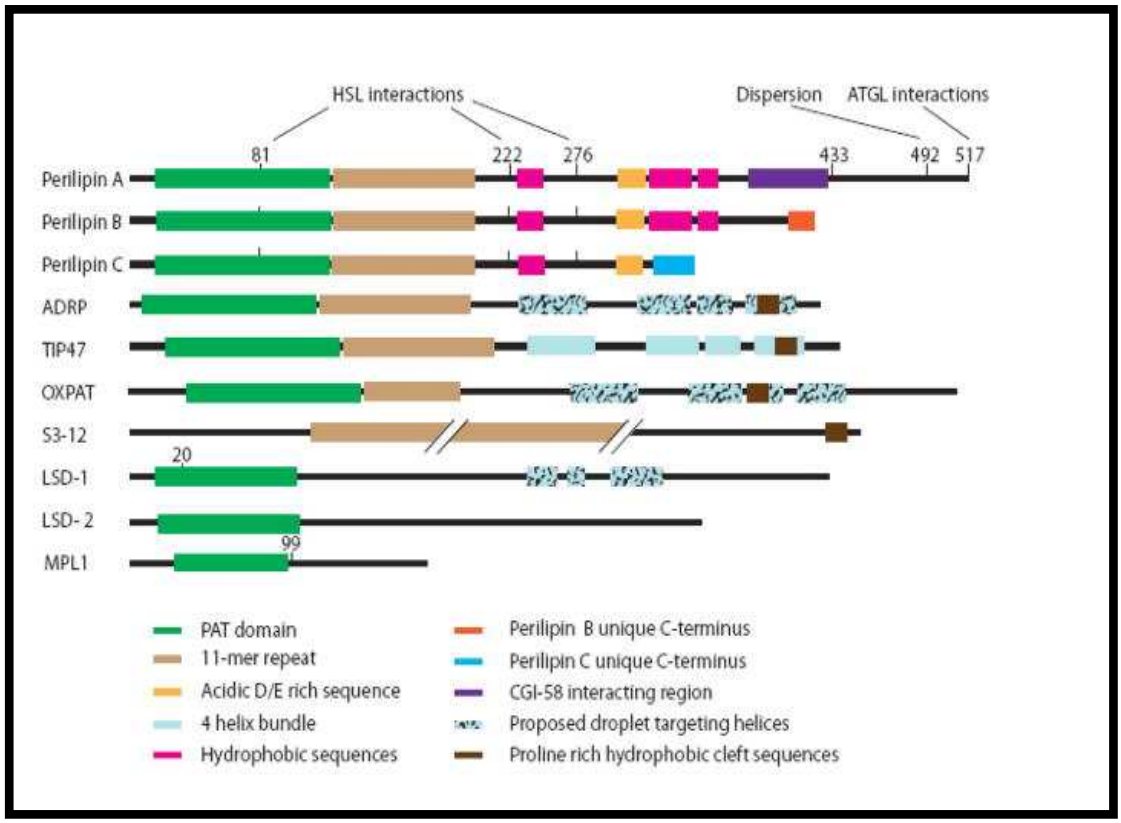

Fig. 4 *PAT family members have similar predicted structural features This cartoon compares the structure of ten members of the PAT family of lipid droplet proteins (Bickel et al. 2009).*

Perilipin is a marker of adipocyte differentiation and thus has been used as a reporter gene to identify regulators of adipogenesis. Expression of the perilipin gene is regulated primarily by peroxisome proliferator activated receptor gamma (PPARγ); however, recent reports also implicate the estrogen receptor related receptor alpha (ERRα) . Furthermore, ERRα-dependent perilipin expression is activated by PPARγ coactivator-1 alpha (PGC-1α) and repressed by the small heterodimer partner (SHP) Perilipin expression also is activated by the constitutive coactivator of PPARγ (CCPG) or repressed by tribbles homolog 3 (TRB3), both acting through PPARγ. By contrast, the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα) induces triacylglycerol (TAG) hydrolysis partially through a decrease in perilipin mRNA and partially through an indirect elevation of cAMP. The effect of TNFα is at least in part mediated by NF-κB and occurs *via* the ERK pathway.

Perilipin associated with droplets has a half-life of 40 hours, while excess perilipin not bound to droplets is rapidly degraded .There is disagreement in the literature as to whether this is due to a proteosomal or lysosomal mechanism. Research laboratory of Londos found that ubiquitinated perilipin accumulates in the presence of the proteosomal inhibitors MG-132 and ALLN in Chinese hamster ovary CHO cells. The Greenberg lab was unable to replicate these results in either NIH-3T3 fibroblasts or 3T3-L1 adypocite cells. Instead, this group found that perilipin degradation was blocked by the lysosomal protease inhibitors leupeptin and ammonium chloride, thereby implicating the lysosomal pathway. Differences in cell type may account for some of the observed dissimilarities. Because all 3 perilipin isoforms are stable only when bound to lipid, the mass of cellular neutral lipid act as a means of post-translational control of perilipin protein levels .

Perilipin A was originally identified as the major protein kinase A substrate associated with the lipid storage droplet . Murine perilipin A is phosphorylated on up to 6 potential PKA sites. It is currently unclear if other kinases play a role in its function. Upon phosphorylation the role of perilipin A shifts from storage to mobilization of stored neutral lipid, as discussed below. Perilipin A is dephosphorylated by protein phosphatase 1. The perilipins are the only mammalian PAT family members whose function is known to be acutely regulated by their phosphorylation state; phosphorylation dependent events have not been reported for ADRP, TIP47, OXPAT, or S3-12.

Lipolysis is the hydrolysis of fatty acyl esters and is the means by which stored lipids are mobilized for production of membranes and for provision of metabolic fuel substrates. For the purpose of energy storage, fatty acids are esterified to a glycerol backbone to generate sequentially mono-, di-, and triacylglycerols, whereas

cholesteryl esters are made to store excess cholesterol for membrane and hormone biosynthesis. In the adipocyte, catecholamines signal through a β-adrenergic receptor and G-protein coupled signaling cascade to elevate cellular cAMP levels . This in turn activates cAMP-dependent PKA, which polyphosphorylates the perilipins and HSL. HSL then translocates from the cytoplasm to the lipid droplet surface (fig.5).

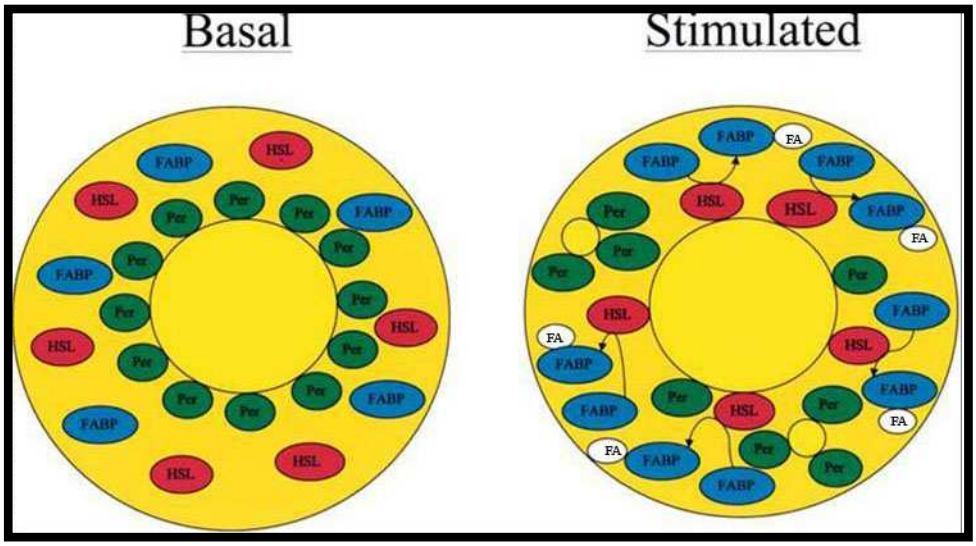

Fig.5 *Schematic representation of the activation of lipolysis by lipolytic hormones.Under basal conditions, perilipin (Per) is located on the surface of the single triacylglycerol droplet, with HSL in the cytoplasm. Upon lipolytic stimulation, both perilipin and HSL become multi-phosphorylated, with perilipin being displaced from the droplet, allowing access for HSL. There is also evidence that fatty acids (FA) are removed from HSL by FABPs, preventing accumulation and resultant product inhibition (Yeaman et al.2004).*

1.6 Hormone sensitive lipase

Hormone sensitive lipase (HSL) is an intracellular neutral lipase that is capable of hydrolyzing triacylglycerols, diacylglycerols, monoacyglycerols, and cholesteryl esters. HSL is responsible for the neutral cholesteryl ester hydrolase activity in steroidogenic tissues. Through its action, HSL is involved in regulating intracellular cholesterol metabolism and making unesterified cholesterol available for steroid hormone production. Steroidogenic acute regulatory protein (StAR) facilitates the movement of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane and is a critical regulatory step in steroidogenesis. It is proposed that the interaction of HSL with StAR in cytosol increases the hydrolytic activity of HSL and that together HSL and StAR facilitate cholesterol movement from lipid droplets to mitochondria for steroidogenesis (*Shen et al. 2003).*

HSL was first recognized as the enzyme controlling lipolysis in white adipose tissue, supported by a monoglyceride lipase (MGL) *(Osterlund. 2001)* The two enzymes work consecutively to hydrolyse triacyl and diacyl glycerol (HSL) and 2-monoacyl glycerol (MGL). At the time of the identification of HSL, it was already known that lipolysis was the subject of hormonal control, and it was the lipase activity that increased with hormonal treatment (and which is rate-limiting) that was given the name hormone-sensitive. Although described initially as an intracellular adypocite–specific triacylglycerol lipase it is now clear is expressed in multiple tissues and plays a number of roles in lipid metabolism, including that of a neutral cholesteyl ester hydrolase. It is highly expressed in adipose tissue an steroidogenic tissue, with lower amount expressed in cardiac and skeletal muscle, macrophages, and islet.

This enzyme has a long and a short shape. The long shape is expressed in steroidogenic tissues such as testis, where it converts cholesteryl ester to free

cholesterol for steroid hormone production. The short shape is expressed in adipose tissue, among others, where it hydrolyzes stored tryglicerides to free fatty acids.

The major isoform is a single polypeptide with a molecular mass of approx. 84 kDa and which comprises three major domains: a catalytic domain, a regulatory domain encoding several phosphorylation sites and an N-terminal domain involved in protein-protein and protein-lipid interactions (fig 6-7) *(Yeaman et al. 2004).*

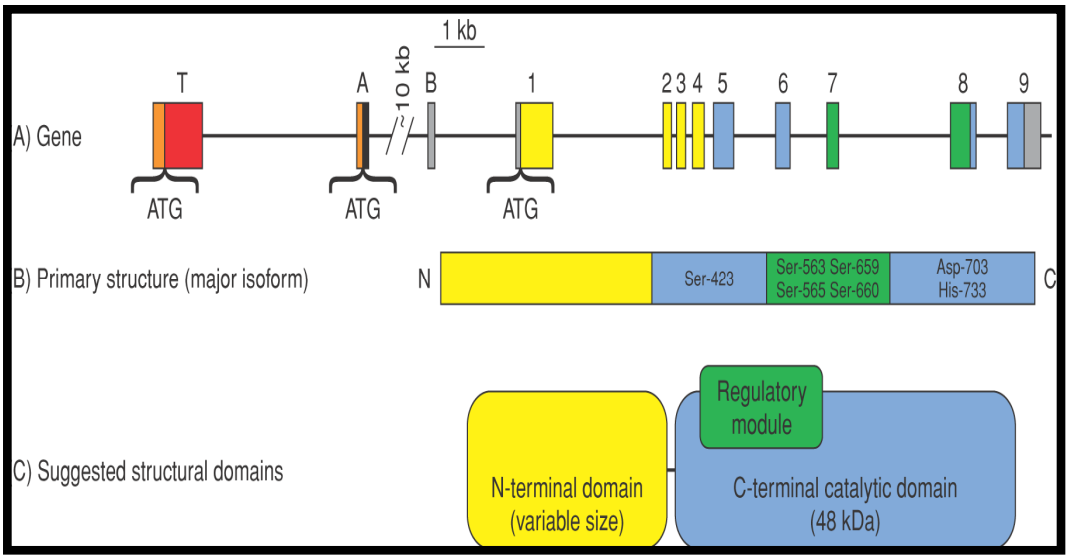

Fig.6 *Outline structure of the human HSL gene and the corresponding protein (Yeaman et al. 2004)*

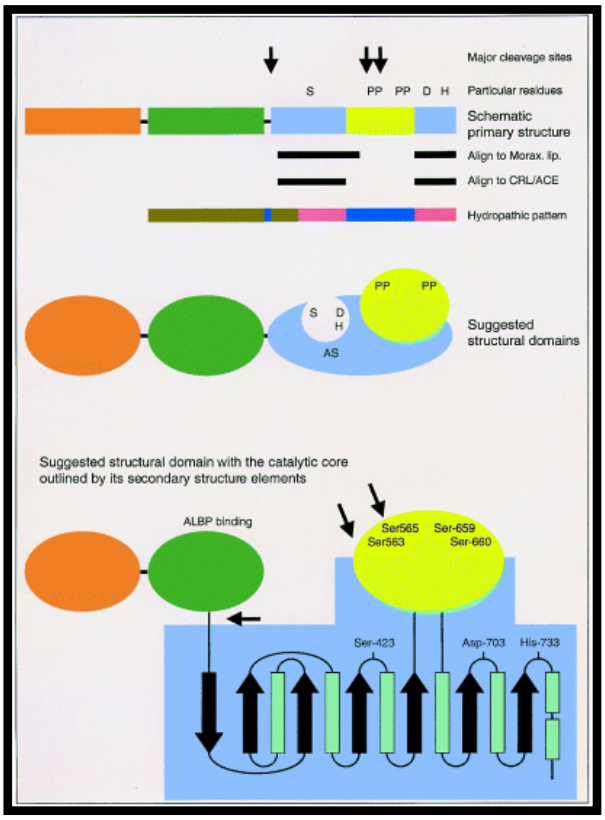

Fig.7 *Structural and functional domains of HSL. A schematic representation of the primary structure of HSL is shown at the top, represented by testis-specific sequence (orange), N-terminal 300 residues (green), catalytic core (purple) and regulatory module (yellow). The major cleavage sites (arrows), important residues of the catalytic triad [Ser423 (S), Asp703 (D) and His733 (H)] and phosphorylation sites (P) (Ser563, Ser565, Ser659 and Ser660) are indicated. The parts that align to the* Moraxella *lipase (Morax. Lip.),* Candida rugosa *lipase (CRL) and acetyl choline esterase (ACE), are shown with bars. Representation of the calculated hydropathic pattern is shown by hydrophilic (blue) hydrophobic (red) and intermediary hydropathy (brown). In the middle are the suggested structural domains with indication of the triad in an active site (AS) and phosphorylation sites. At the bottom is the suggested structural domains with a representation of the catalytic core outlined by its secondary structure elements (β strands by black arrows and α helices by light green rectangles). The testis specific sequence and domain is only found in* HSL_{tes} *whereas the rest can be found in both* HSL_{tes} *and* HSL_{adi} *(Osterlund. 2001)*

One of the unique features of HSL that differentiates it from most other lipases is that its activity against triacylglycerol and cholesteryl ester substrates appears to be regulated by reversible phosphorylation; however, hydrolytic activity against diacylglycerol, monoacylglycerol and water-soluble substrates is unaffected by

phosphorylation .PKA increases the hydrolytic activity of HSL by phosphorylation of a single site that was initially identified as S563 in rat HSL and is located within the regulatory module. Although evidence to support the phosphorylation of S563 by PKA has been provided from mutagenesis experiments , other investigators have reported that S659 and S660 were phosphorylated by PKA in vitro and were required for the phosphorylation-induced increase in hydrolytic activity against triacylglycerol substrate. Additionally, lipolytic hormones not only can activate PKA, but also the mitogen activated protein kinase pathway and extracellular signal-regulated kinase (ERK). Activation of the ERK pathway appears to be able to regulate adipocyte lipolysis by phosphorylating HSL on S600 and increasing the activity of HSL.In contrast to activation of activity seen with PKA or ERK phosphorylation, other kinases such as glycogen synthase kinase-4, Ca^{++}/calmodulin-dependent protein kinase II, and AMP-activated protein kinase phosphorylate HSL at a secondary basal site S565 in rat HSL . Phosphorylation at S565 impairs the phosphorylation of S563 by PKA . HSL activity can be inactivated by protein phosphatases. The most active phosphatases against S563 are phosphatase 2A and 2C, while S565 is predominately dephosphorylated by phosphatase 2A . Thus, several different kinases phosphorylate HSL at unique serines within the regulatory module and modulate HSL activity *(Kraemer et al. 2000)* The hydrolytic action of HSL is regulated by perilipin A, a lipid droplet-associated protein. Association of perilipin A with lipid droplet controls the magnitude of lipolysis. This perilipin A may act as a barrier to lipases, thereby maintaining a low rate of basal lipolysis. Upon hormonal stimulation, perilipin A undergoes phosphorylation by PKA at six serine residues, and phosphorylation at Ser-517 may globally regulate PKA-stimulated lipolysis in adipocytes. PKA-dependent perilipin phosphorylation may facilitate the translocation of HSL to the

lipid droplet. However, one recent study showed that PKA-dependent perilipin phosphorylation, although facilitating perilipin interaction with lipid droplet-associated HSL, is not involved in the translocation of HSL to the lipid droplet *(Jaworski et al. 2007)*

1.7 Conjugated Linoleic Acids

Conjugated linoleic acids (CLA) represent a heterogeneous group of positional and geometric isomers of linoleic acid, which are predominantly found in milk, milk products, meat and meat products of ruminants and it is present in dietary supplements mainly taken by physically active subjects. The characteristic of these isomers is that, unlike linoleic acid, the two double bonds along the 18-carbon fatty acid chain are separated by a single carbon-carbon bond, not a methylene group (fig. 8) The biological activities attributed to CLAs have to be confirmed, if such attributes are due to a single isomer or of the mixture. Being the predominant isomers, cis-9, trans-11-CLA (9-CLA, the rumenic acid) and trans-10, cis-12-CLA (10-CLA) are the primary focus of most of the studies evaluating the biological activities of CLA which are primarily derived from linoleic acid, a typical n-6 fatty acid. Like neutraceuticals, being minor lipids with supposed functional food status, CLA are getting momentum in alleviating major killer diseases such as cancer, atherosclerosis, and diabetes in humans *(Benjamin et al. 2009).*

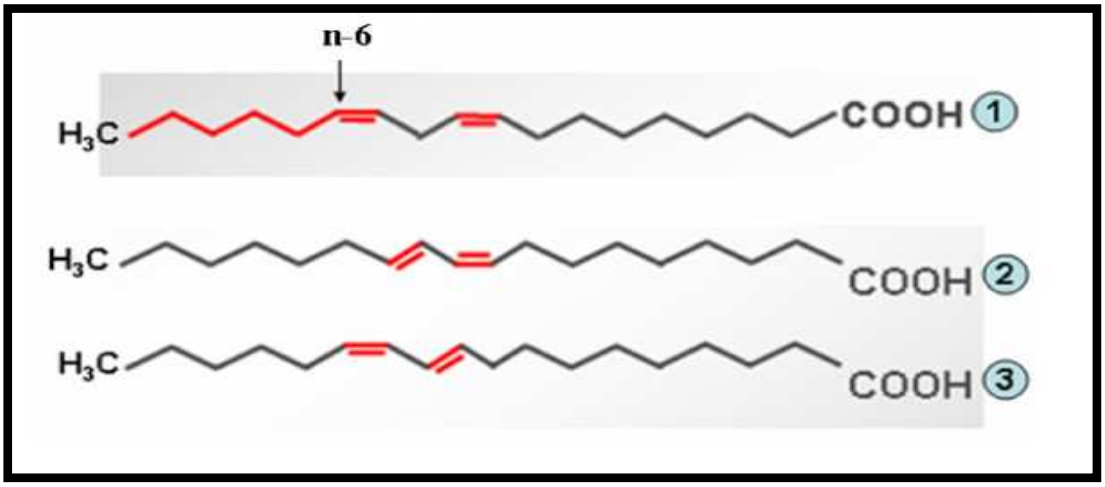

Fig.8 *Structure of linoleic acid and its major CLA derivatives. 1. Linoleic acid (typical n-6 PUFA); 2.* cis-*9,* trans-*11-octadecadienoic acid (9-CLA, in fact an n-7 fatty acid); 3.* trans-*10,* cis-*12-octadecadienoic acid (10-CLA) (Benjamin et al. 2009).*

The use of CLA supplement is spreading amongst elite and recreational athletes because marketing claims suggest that it should improve endurance capacity, increase VO_2max, reduce body mass, reduce muscle glycogen breakdown, improve metabolism, and prevent or reduce muscle damage and inflammatory response, promote fat loss and reduce catabolism *(Jeukendrup et al. 2004; Kreider et al. 2002).* Few studies have been conducted to evaluate the effects of CLA supplementation and exercise training (endurance and resistance) in animal and human subjects. In animals, it has been demonstrated that CLA supplementation associated with endurance training decreases body weight *(Faulconnier et al. 2004)* and adipose tissue *(Bhattacharya et al. 2005, Faulconnier et al. 2004, Thom et al. 2001)* and increases lean body mass (*Bhattacharya et al. 2005*), muscle hypertrophy (*Di Felice et al. 2007*) and endurance performance (*Mizunoya et al. 2005*). Although the results in animals show that CLA supplementation has positive effects, the studies conducted on humans show contradictory results. Kreider et al. (*Kreider et al. 2002*)

and Pinkoski et al. (*Pinkoski et al. 2006*), who investigated the effects of CLA supplementation during resistance exercise training on subjects performing different sporting activities, observed opposite results in spite of the fact that both performed randomised controlled studies. CLA supplementation in experienced resistance-trained athletes did not induce differences in total body mass, body composition and strength (*Kreider et al. 2002*), while in moderately physically active subjects, it induced a relatively small increase in lean body mass and a decrease in body fat mass (*Pinkoski et al. 2006*). In *in vitro* studies, the lipolytic effect of CLA on adipocytes seems to be mediated by an increase in the levels of the perilipin A protein (*Chung et al. 2005*). Degner et al. suggested that coniugated linoleic acid attenuates cyclooxygenase-2 activity *(Degner et al. 2006)* and that inhibition increases steroid production *(Wang et al. 2003; Chen et al. 2007).*

2. Aims of the project

The effect of CLA supplementation on body fat mass reduction has been studied (*Campbell et al. 2008*), but there are no studies that explain at the molecular level why CLA may induce this effect. In *in vitro* studies, the lipolytic effect of CLA on adipocytes seems to be mediated by an increase in the levels of the perilipin A protein (*Chung et al. 2005*). The proteins of the perilipin family are found exclusively on the outer surface of lipid storage droplets linked to the hormone-sensitive lipase in adipocytes or to the cholesteryl esterase in steroidogenic cells on the cytoplasmic side, and these proteins provide a protective coat on the lipid droplet surface. In steroidogenic cells there are two isoforms of perilipin, the A and the C isoforms. Upon stimulation, both perilipin and cholesteryl esterase become multi-phosphorylated, with perilipin being displaced from the droplet allowing the cholesteryl esterase access. The steroidogenic cells cholesteryl esters, precursors of steroid hormones, are stored in intracellular lipid droplets and their hydrolysis is activated by protein kinase A, which phosphorylates the perilipins, and is catalysed by a cholesteryl esterase similar to the hormone-sensitive lipase expressed in adipocytes (*Servetnick et al. 1995*). Moreover it has previously been suggested that exercise training might alter the amount and/or function of perilipin, thereby regulating the interaction of hormone-sensitive lipase with its substrate (*Nomura et al. 2002*). Considering the lipolytic effect of CLA, the role of perilipins in the pathway leading to steroid hormone synthesis and the effect of exercise on perilipin function, we hypothesised that CLA should increase the levels of testosterone in the blood of physically active subjects after a resistance exercise bout. We also hypothesised that this effect is mediated by the expression and the phosphorylation state of the perilipin proteins. Because it is impossible to determine the level of

perilipins in humans through a biopsy, the hypothesised pathway was studied in a Leydig cell line, a physiological model for studying steroid biosynthesis in vitro that produces testosterone at basal conditions, simulating the activation of these cells in humans after a resistance exercise bout.

We chose a resistance exercise protocol because it involves a large muscle mass at high intensity simulating training of recreational gymnasium users, differently from other protocols proposed in literature, because it increases (around 30%) the total testosterone concentration in men until the end of the resistance exercise bout *(Tremblay et al. 2004; Vingren et al. 2008).* Therefore the aim of the present study is to investigate of the influences of CLA supplementation on testosterone levels *in vivo* in the blood of physically active subjects after a resistance exercise bout and *in vitro* on the perilipin and HSL proteins expression in a tumour cell line derived from rat Leydig cells (R2C).

3. Experimental procedures

3.1 Subjects

Ten physically active male subjects (age: 27.4±3.7 years; height: 176.7±5.2 cm; weight: 81.1± 9.8 Kg; BMI: 25.9±2.6: body fat: 11.9±3.6%) volunteered to participate in this investigation. Each had more than 2 years of resistance training experience and had been training at least three times per week over the last year. All subjects completed a health history questionnaire, were healthy and had no medical contraindications or history of any endocrine disorders. The subjects were not vegetarians, nor did they follow a carbohydrate-restricted diet. No subjects were taking any medications or nutritional supplements, and all were non-steroid users and non-smokers. Subjects were informed of the experimental procedures and associated risk before providing written informed consent. This study is conformed to the standards set by the latest revision of the Declaration of Helsinki, and it was approved by the institutional review board for the protection of human subjects of the University of Palermo.

3.2 Experimental design

This study was conducted as a crossover, double-blind, placebo controlled clinical trial. The subjects were randomised to groups receiving either CLA (see below for dosage) or placebo (PLA) for 21 days. After 2 weeks of washout, the groups were switched and all subjects received the opposite substance for an additional 21 days. Before and after each supplementation period, the subjects underwent body composition analysis and a resistance exercise bout (fig.9). During the 6 weeks of supplementation, the subjects were instructed to consume their regular diet, to

maintain their usual training routine and to maintain normal nocturnal sleep habits (i.e., 7-8 h/night). It was recommended that they abstain from vigorous exercise, alcohol use and sexual activity for at least 24 hours before the resistance exercise.

3.3 CLA and PLA supplementation

The subjects ingested 7 opaque soft gel capsules of CLA (6 g of Tonalin® FFA 80, Cognis Group, Germany) or PLA (6 g of sunflower oil) per day, both identical in taste and in appearance. The CLA supplement was a mixture containing 40% c9,t11-CLA and 40% t10,c12-CLA. The rest of the mixture was made of other fatty acids (10% of oleic acid, < 4% of palmitic acid, < 4% of stearic acid and < 3% of linoleic acid). At the beginning of each supplementation period, the subjects received three boxes with 56 pills each. At the end of each supplementation period, the remaining pills per box were counted to verify the supplement intake. Subjects consumed 99.6±1.3% and 99.2±1.7% of the prescribed intake during the CLA and PLA supplementation periods, respectively.

3.4 1-RM strength test

Maximal strength was determined for each of the eight exercises of the resistance protocol. This was assessed by completing a one-repetition maximal (1-RM) strength test (i.e., the heaviest load that can be lifted one time through a full range of motion). Three to four subsequent attempts were performed to determine the 1-RM strength, with the weight increasing progressively until the subjects failed at the given load. The 1-RM strength test was preceded by a warm-up session consisting of 10 min cycling on a stationary bike, 5 min light stretching and 2-3 sets of 8-10 repetitions of

leg press at < 50% of perceived maximal strength. Baseline testing was performed one week prior to the start of supplementation.

3.5 Resistance exercise bout protocol

The resistance exercise bout consisted of 8 resistance exercises (leg press, leg curl, leg extension, lat pull-down, bench press, shoulder press, barbell bicep curl and supine triceps extension). The subject's goal was to complete three sets of 8 to 10 repetitions at approximately 75% of that subject's 1-RM strength. One minute of rest between each set and 2 min between each exercise were allowed for recovery. The number of repetitions and the amount of weight lifted in each exercise were recorded to calculate the total lifting volume, i.e. the training volume. A warm-up was performed before each resistance exercise bout. The resistance exercise bouts and the 1-RM strength test were performed under the supervision of qualified personal trainers.

3.6 Body composition analysis

Body composition analysis was performed early in the morning after a night of fasting on the first and the last day of each supplementation period with the Inbody 320 (BioSpace, Seoul, Korea), a bioelectrical impedance analyser. The device uses eight points of tactile electrodes (contact at the hands and feet) and three frequencies (5, 50 and 200 kHz) to measure body mass (kg), body fat mass (kg), lean body mass (kg) and intra- and extra-cellular water *(Jensky-Squires et al. 2008).*

All subjects were asked to empty their bladders immediately before the measurements. The bioelectrical impedance analysis was performed with subjects dressed only their underwear (with all jewellery removed). The validity and

reliability of this specific bioelectrical impedance analyser in adult males has been tested by Jensky-Squires et al. (*Jensky-Squires et al. 2008*).

3.7 Blood sampling and analysis

Each resistance exercise bout was performed at 4:00 pm after a 4 h fast, to minimise the influence of diurnal variations on exercise performance and hormonal response *(Bird et al. 2004)*. Blood was drawn before (Pre) and after (Post) each resistance exercise bout from the antecubital vein to determine hormone concentrations (total testosterone, estradiol and cortisol) and sex hormone binding globulin (SHBG) levels. A butterfly needle was inserted into an antecubital forearm vein, after which subjects sat quietly for 15 min. The Pre blood samples were drawn after another 15 min, in which subjects sat quietly to minimise hormonal fluctuations related to anticipatory responses. The Post blood samples were drawn immediately after completion of the resistance exercise bout (*Bird et al. 2004*). Blood measurements were performed by the haematology laboratory of "Azienda Ospedaliera Villa Sofia – CTO Palermo". The hormones and SHBG were measured on an Immulite 2000 (Medical System S.P.A., Genova, Italy) using a method and an assay that have been fully described previously (*Elmlinger et al. 2005*).

3.8 Cell cultures

Rat Leydig tumour cells (R2C, cat. No. 89031606, ECACC, Health Protection Agency Culture Collections, Salisbury, UK) **(**fig. 13) were cultured in 75-cm^2 flasks in M-199 medium (Invitrogen Corp., Carlsbad, CA) supplemented with 15% horse serum (Invitrogen Corp.), 2.5% fetal bovine serum (Invitrogen Corp.) and antibiotic antimycotic solution (penicillin 100U/ml, streptomycin 100µg/ml, Amphotericin B 0.25 µg/ml; Invitrogen Corp.). Cells were incubated at 37°C in a humidified

atmosphere with 5% CO_2 and fed three times a week until reaching 80-90% confluence. Adherent cells were harvested with trypsin in Ca^{2+} and Mg^{2+} free Hanks' Balanced Salt Solution (HBSS, Invitrogen Corp.), washed with phosphate-buffered saline and resuspended in the supplemented M-199 medium. Cells were seeded at $5x10^3$ cells/well in 96-well plates for (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) Assay (MTS assay) at $5x10^4$ cells/well in 24-well plates for the testosterone secretion determination, at $15x10^3$ cells/well in 8-well chamber slide for the immunofluorescence and at $7x10^5$ cells in 25-cm^2 flasks for Western Blotting. When cells reached about 80% of confluence, they were treated with the CLA working solution at increasing concentrations (0- to 30 µM) for 24h and 48h. The concentrations used were tested with a tetrazolium compound colorimetric assay (MTS assay) and they did not induce any changes in cell viability. To prepare the CLA working solution, the Tonalin® FFA 80 (Cognis, was first dissolved in absolute ethanol and then in fetal bovine serum containing 1% bovine serum albumin (Sigma – Aldrich, St. Louis, MO). To determine testosterone secretion, the medium was collected at the end of the incubation period and subsequently analysed with an Immulite 2000 (Medical System S.P.A.).

3.9 (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) Assay

For the MTS Assay, the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega Corporation) was used following the manufacturer's instruction. Briefly after the incubation period of 24h and 48h with the CLA working solution, 20 µl of the MTS reagent was added into each well and cells were incubated at 37°C for

3h. At the end of the incubation period the absorbance was detected at 490 nm with a multiwall-plate reader (GDV – DV990BV4). The quantity of formazan product was measured at 490 nm, and it is directly proportional to the number living cells in culture.

3.10 Immunofluorescence staining

After removing the media, the attached cells were sequentially washed with PBS (2 X 5 min) and fixed in 4% paraformaldehyde in PBS (15 min) and ice cold methanol (30 min). Next they were washed (3 X 5 min), and incubated in unmasking solution (Tri-sodium citrate buffer and 0.05% Tween20) for 10 min, washed (3 X 5 min) and blocked in T-TBS containing 3% Bovine Serum Albumin. Next, anti-Perilipin A/C (1:50, a goat polyclonal antibody that was a gift from Prof. Londos - National Institutes of Health, Bethesda, USA) and anti-HSL (1:50, a rabbit polyclonal antibody. PRS3965, anti-Lipe, Sigma-Aldrich) were added and the cells were incubated in a humidified chamber overnight at 4°C. The cells were then washed with PBS (3 X 5 min) and incubated for 1 h at RT with anti-goat FITCH- conjugated secondary antibody (Sigma-Aldrich) diluted 1:200 and anti-rabbit TRICH-conjugated secondary antibody (Sigma-Aldrich) diluted 1:100 The cells were then washed in PBS (3 X 5 min) and incubated with HOECHT33342 (Sigma-Aldrich) diluted 1:1000 for 15 min. Samples were observed with a direct fluorescence Leica Microscope DM 5000 CTR.

3.11 Western Blotting analysis

After the incubation period, the medium was removed, cells were washed twice with ice-cold PBS, and then they were lysed with a 100 µl lysis buffer (200 mM Hepes,

5M NaCl, 10% Triton-x100, 0.5M EDTA, 1M DTT, 0.25g Na-deoxycolate, 0.05g SDS) on ice for 30 min and then centrifuged at 15900g for 10 min at 4°C. Equal amounts of proteins measured by the Bradford method, 55 µg/lane for Perilipin A/C and 40 µg/lane for HSL, were respectively separated on a 12% SDS-PAGE gel. The separated proteins were electrophoretically transferred to a nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences, GE Healthcare, Little Chalfont, England) and incubated with a blocking solution containing 5% dry milk in Tris-Buffered Saline (20mM Tris, 137mM NaCl, pH 7.6) with 0.05% Tween20 (T-TBS) for 1h at RT. Perilipin A/C protein levels were detected by immunoblotting using a goat polyclonal antiserum specific for Perilipin A/C (anti-Perilipin A/C) diluted 1:500 in T-TBS containing 1% dry milk and HSL protein levels were detected by immunoblotting using a rabbit polyclonal specific for HSL (anti-Lipe, PRS3965, Sigma-Aldrich) diluted 1:1000 in T-TBS containing 1% dry milk. Blots were washed in T-TBS and incubated respectively for 1 h with a secondary antibody of conjugated donkey anti-goat IgG-horse radish peroxidase (diluted 1:8000 in T-TBS containing 1% dry milk, S.Cruz Biotechnology) and HRP-conjugated secondary antibody diluted 1:10000 in T-TBS containing 1% dry milk (ECL^{TM} anti-rabbit IgG HRP-conjugated whole antibody, Amersham Biosciences). The final detection procedure was performed using the ECL Western Blotting Detection Reagent (Amersham Biosciences) according to the manufacturer's instructions. Band analysis was performed with the ImageJ 1.41 software (National Institutes of Health, USA, http://rsb.info.nih.gov/ij). The phosphorylated state of the perilipin proteins was determined by visualisation of a delayed band in the Western Blotting analysis according to Servetnick et al. (*Servetnick DA* et al.1995). An antibody against the phosphorylated form of perilipins is not commercially available.

After perilipin or HSL detection, filters were stripped in TRIS-HCL 20mM pH7.6 containing 137 mM NaCl, 0,01% β Mercaptoethanol and detected again for the expression of actin diluted 1:1000 in T-TBS, anti-actin, (AC-40, Sigma-Aldrich).

3.12 Cell Based ELISA

For coating cells were cultured in 96-well plates, doubling the number of cells ($0{,}16x10^3$ to $10x10^3$ cells/well) in each consecutive well to obtain an expression curve. Perilipin A/C and HSL antibodies were tested twice per plate. Twenty –four hours after plating, the cells were treated with the CLA working solution at increasing concentrations (0- to 30 µM) for 48h. After the incubation period the cells were fixed with ice –cold methanol for 30 min. After fixation, the cells were rinsed three-times in PBS and blocked in PBS containing 5% Bovine Serum Albumin for 1h. Next the single protein were detected with primary antibodies diluted 1:500 in PBS containing 5% Bovine Serum Albumin (anti-perilipin A/C; anti-HSL PRS3965, Sigma-Aldrich) for 1h at room temperature. After washing, primary antibodies were detected with anti-goat, anti-rabbit HRP-conjugated secondary antibodies diluted 1:3000 in PBS containing 5% Bovine Serum Albumin respectively for 1h. After washing SIGMAFAST o- Phenylenediamine dihydrochloride (OPD) Tables (Sigma-Aldrich) were used as HRP substrate and read at 495 nm.

3.13 Statistics

Body composition measurements and mean training volumes were analysed by one-way ANOVA for repeated measures: Trials (Before PLA, After PLA, Before CLA and After CLA). Hormones and SHBG measurements were analysed by two-way ANOVA with repeated measures: Trials *vs* resistance exercise bouts (Pre and Post).

One-way ANOVA analysis for different CLA concentrations was performed on perilipin A/C expression and testosterone secretion by R2C cells. If a significant difference was detected during one- or two-way ANOVA analyses, this was further evaluated by post-hoc Tukey test. Statistical analyses were performed using SigmaStat (Heame Scientific Software, Chicago, IL). All data are presented as means ± SE, and the level of statistical significance was set at $p<0.05$.

4. Results

4.1 Training volume

No significant differences were observed among the trials in sum or with each exercise lifting volume (tab.1).

4.2 Body composition

The absolute data on body composition are shown in figure 10A. There were no significant differences in total body mass, body fat mass and lean body mass Before and After the CLA and PLA supplementation (p=0.212, 0.666, 0.068 respectively). Considering only the changes before and after supplementation, an increase in the lean body mass (0.67±0.6 Kg) was observed in CLA treated subjects (figure 10B). No difference was observed in intra- and extra-cellular water (data not shown).

4.3 Serum hormones and sex hormone –binding globulin

The serum hormones and SHBG concentration results are shown in figure 11. The testosterone and SHBG concentration increased significantly after exercise (p=0.030 and p=0.042, respectively). In particular, the testosterone value Post resistance exercise bout After CLA supplementation was significantly higher than the corresponding Pre resistance exercise bout value. The estradiol and cortisol plasma levels did not change in relation to Trials and/or resistance exercise bouts.

4.4 Testosterone production in vitro

To test if the increase in the blood levels of testosterone depends on the direct effect of CLA on the Leydig cells present in the testis, we decided to treat *in vitro* R2C rat Leydig cells with different concentrations of CLA (fig. 12). To decide which concentration to use, we tested the viability of R2C cells without or with different concentrations of CLA (fig. 13). CLA induced cell death at concentrations higher than 15 µM after 48h (30-40 % of mortality) (fig.13). Hence we decided to use concentrations up to 30 µM. At the same time the supernatants of twin plates were used to test the concentration of testosterone released in the culture medium.

We did not observe any difference among the different concentrations within 24h, while CLA induced an increase in the secretion or synthesis of testosterone only 48h from the beginning of the treatment. The concentration of testosterone in the medium was higher at 7.5 µM CLA, and it decreased soon after probably due to the loss in viability of the same cells at higher concentrations (fig.14).

4.5 Perilipin A/C expression

One of the proteins involved in the synhtesis of testosterone by Leydig cells is perilipin. It can be phosphorylated by the HSL proteins, as I previously discussed in the introduction of the present thesis. Hence we tested the expression levels of perilipin and its phosphorylation state by Western Blotting, and its expression levels by immunofluorescence and cell-based ELISA. Figure 15-16 shows the perilipin A/C expression in R2C cells with immunofluorescence and Western Blotting analysis. No marked differences in the perilipin A/C expression were observed in immunofluorescence experiments on R2C cells treated with different CLA

concentrations for 48h compared to control. Since immunofluorescence experiments do not distinguish between the A and C isoforms or their phosphorylation state, a Western Blotting analysis was performed. There are no differences both in the amount and in the phosphorylation state of perilipin A/C protein in R2C cells treated with different concentrations of CLA at 48 h when compared to untreated cells. There were no differences also at 24h. That the expression levels of perilipin did not change upon CLA treatment was confirmed in cell-based ELISA (fig.17).

4.6 Hormone sensitive lipase (HSL) expression

HSL is directly correlated to the phosphorylation of perilipin. Immunofluorescence experiments and Western Blotting analysis showed that HSL expression levels did not change with different concentrations of CLA after 48h (fig.18-19). These data are confirmed in cell-based ELISA where no differences were observed in the HSL expression at 48h treatment (fig.20).

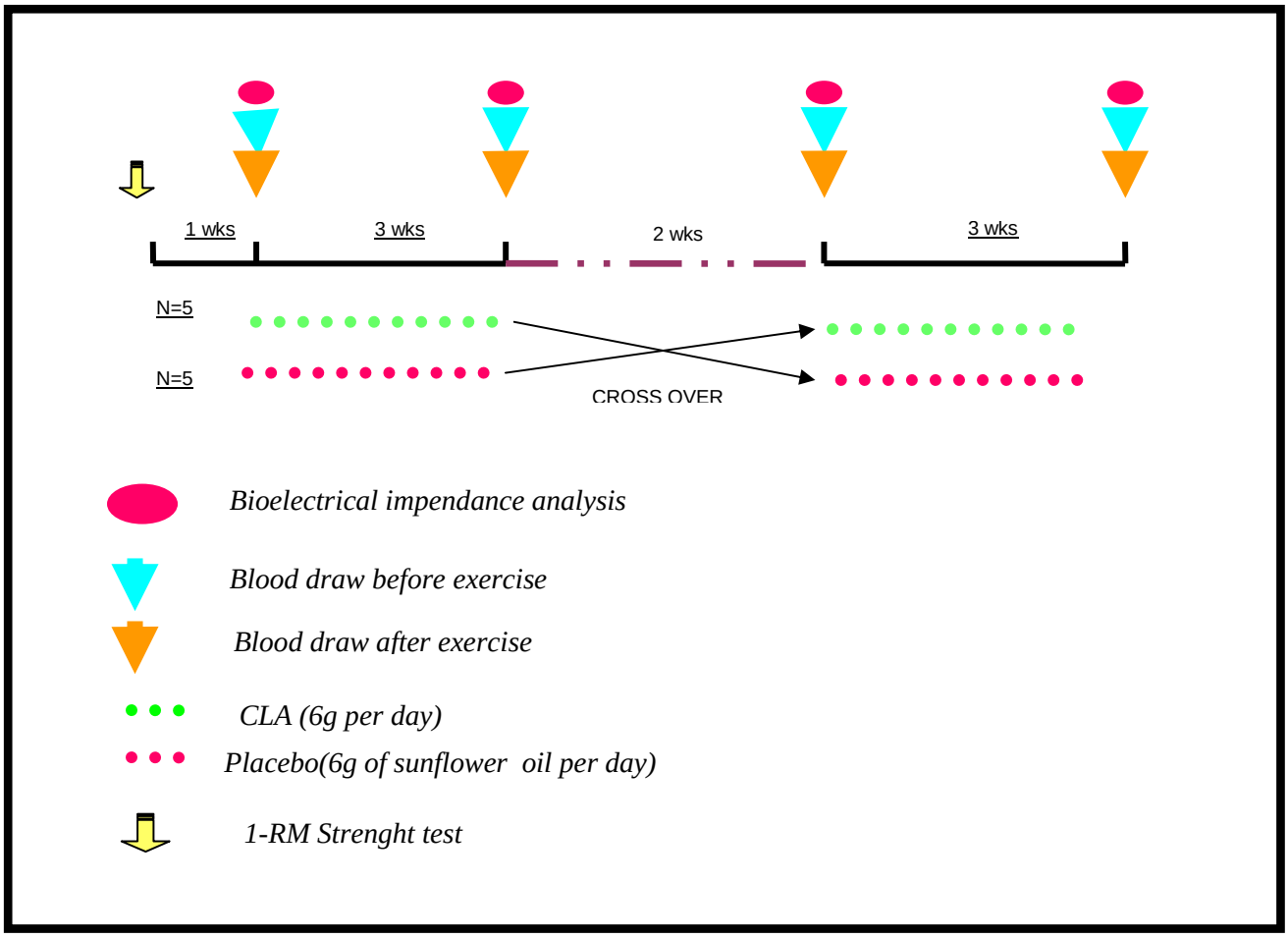

Fig.9 *Experimental design.*

	Before PLA	After PLA	Before CLA	After CLA
Leg press (kg)	4031±1233	4253±1128	3912±1179	4193±1547
Leg curl (kg)	926±215	1023±284	927±210	942± 239
Leg extension (kg)	1203±317	1307±353	1382±622	1373± 675
Lat pull-down (kg)	2033±450	1988±478	1959±554	2161± 354
Bench press (kg)	2296±559	2126±631	2222±557	2311± 542
Shoulder press (kg)	1646±741	1830±787	1551±670	1659±423
Barbell bicep curl (kg)	952±217	1003±202	943±203	951±209
Supine triceps extension (kg)	939±274	1051±447	1015±420	1046±431
Total volume (kg)	13637±3223	14222±3318	14306±3671	15170±3851

Tab.1 *Training volume. All values are means ± SD. PLA indicates placebo supplementation; CLA, conjugated linoleic acid supplementation.*

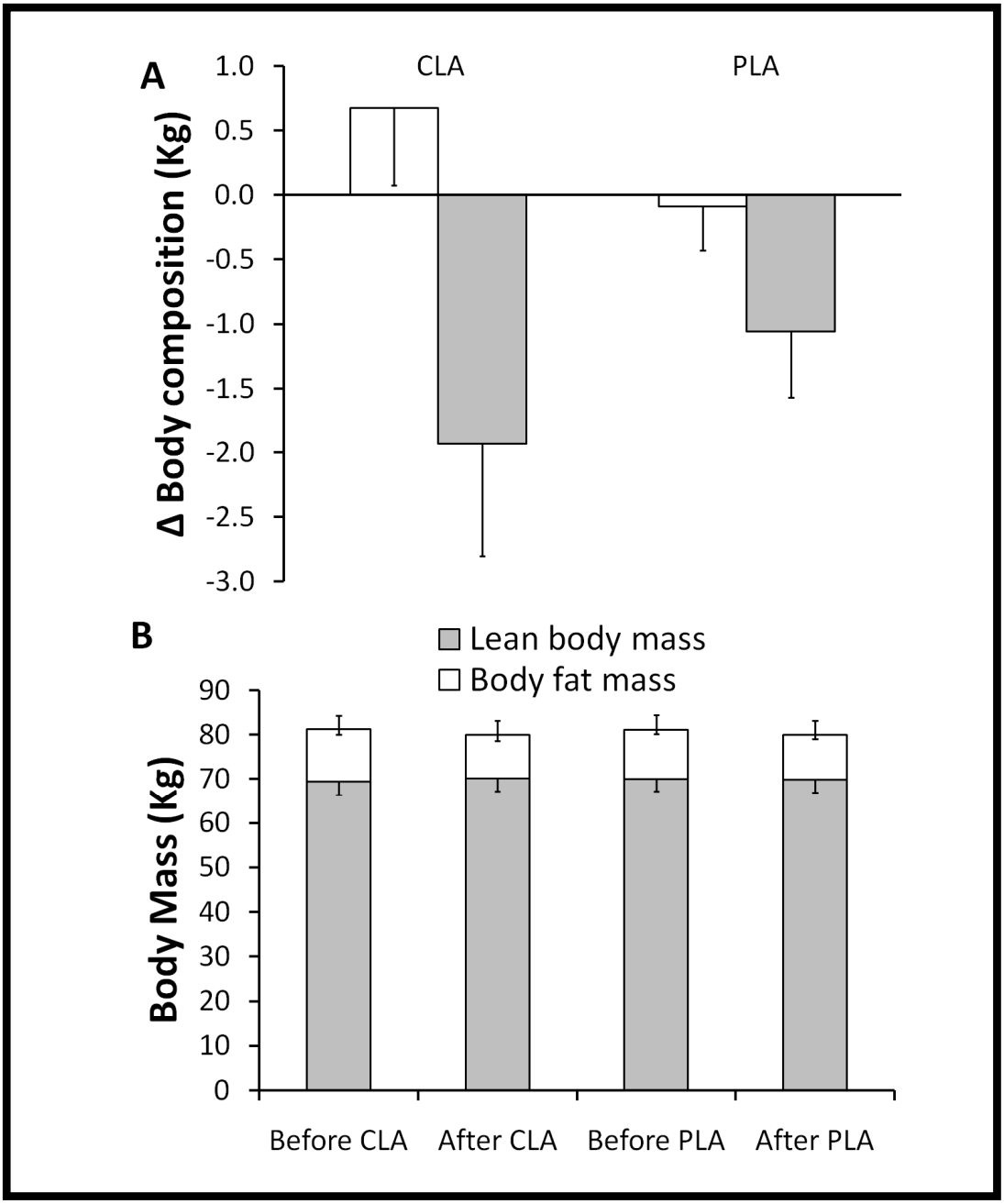

Fig.10 *Body composition: absolute data (A) and changes before and after supplementation (B). All values are means ± SE. PLA indicates placebo supplementation; CLA, conjugated linoleic acid. Standard errors are indicated with error bars, above for total body fat mass and below for lean body mass and body fat mass.*

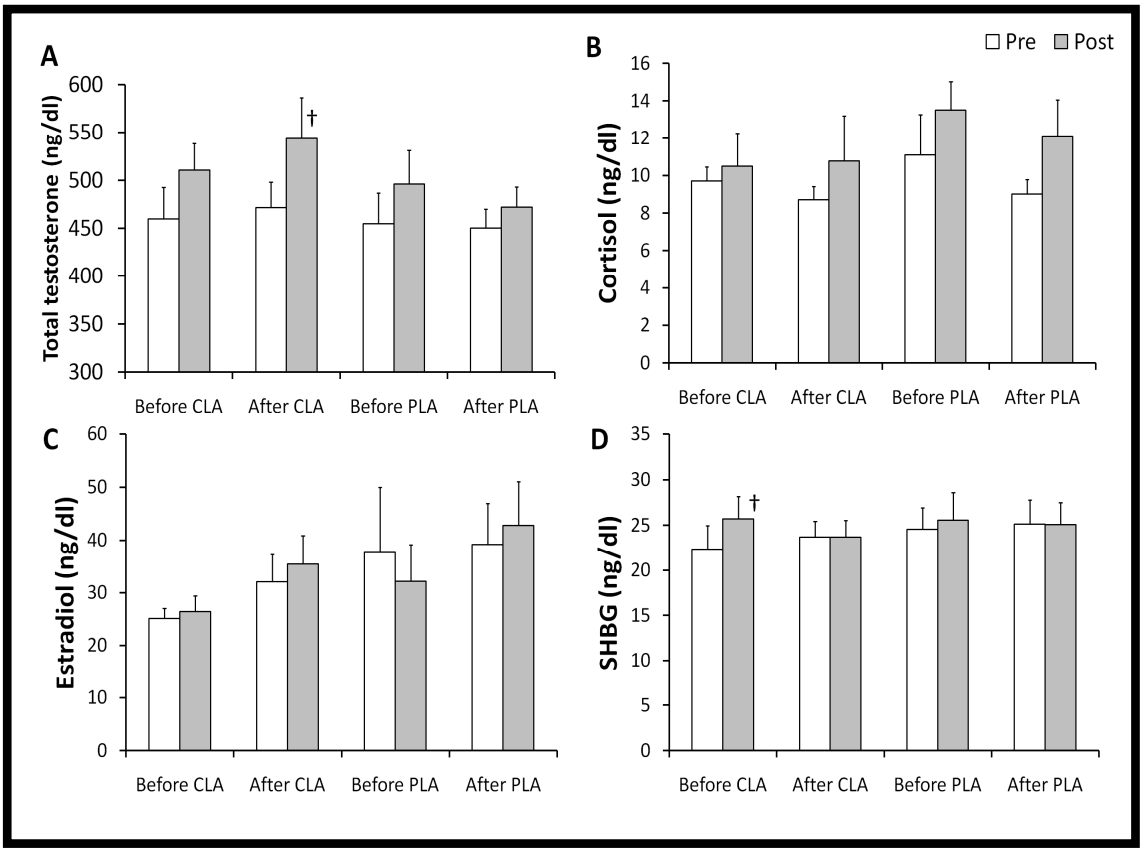

Fig.11 *Serum hormones and sex hormone binding globulin (SHBG). All values are means ± SE. PLA indicates placebo supplementation; CLA, conjugated linoleic acid supplementation; Pre, before resistance exercise bout; Post, after resistance exercise bout. † significant different than the corresponding Pre value ($p<0.05$).*

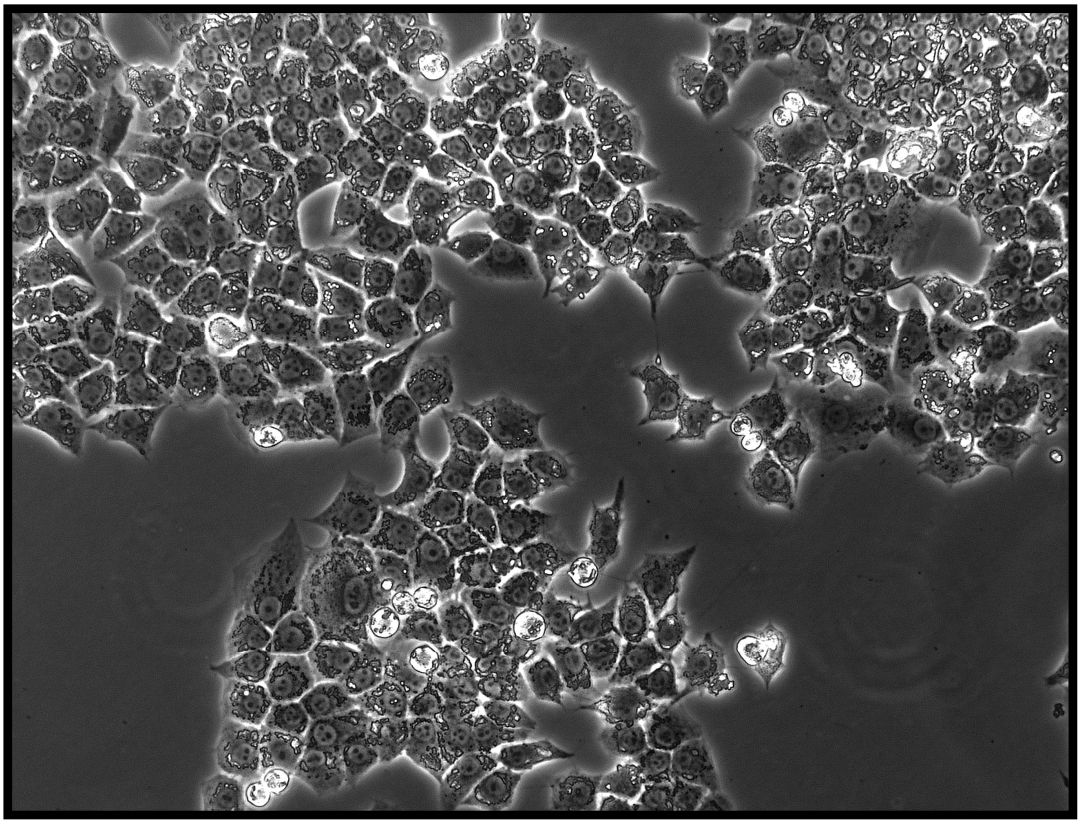

Fig.12 *Phase contrast image of Rat Leydig tumour cells (R2C) cultured on plasticware.*

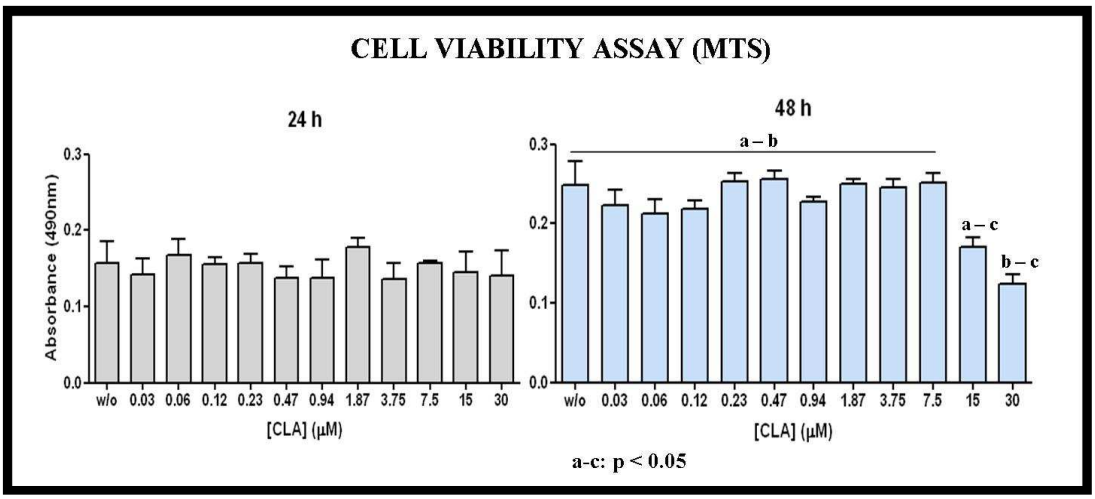

Fig.13 *Diagram of cell viability (MTS assay) of R2C cells after treatment with different concentrations of CLA (0 to 30μM) for 24h and 48 h.*

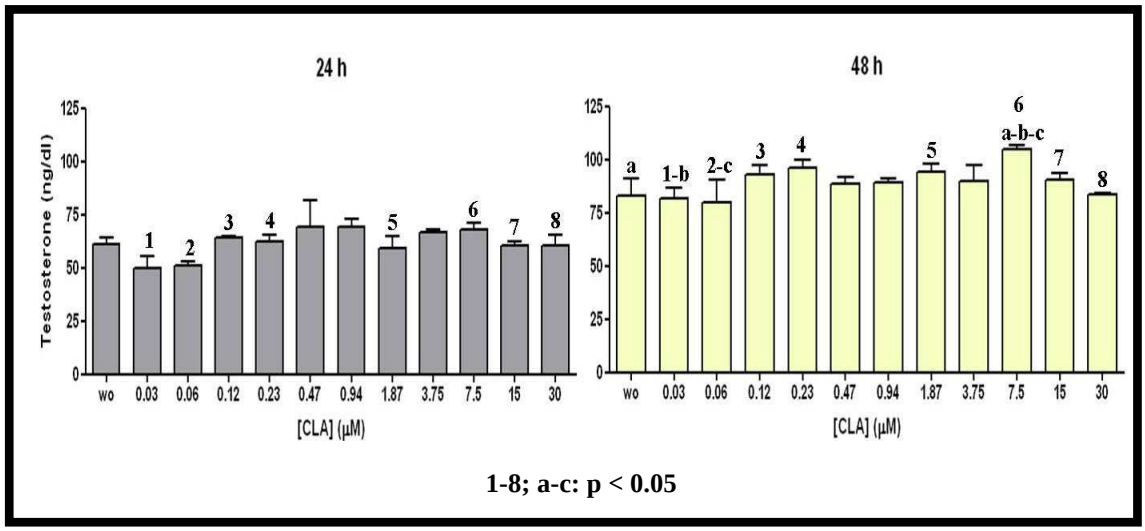

Fig.14 *Testosterone production in R2C cells at 24h (gray bars) and 48h (yellow bars) at different CLA concentrations. All values are means ± SE.*

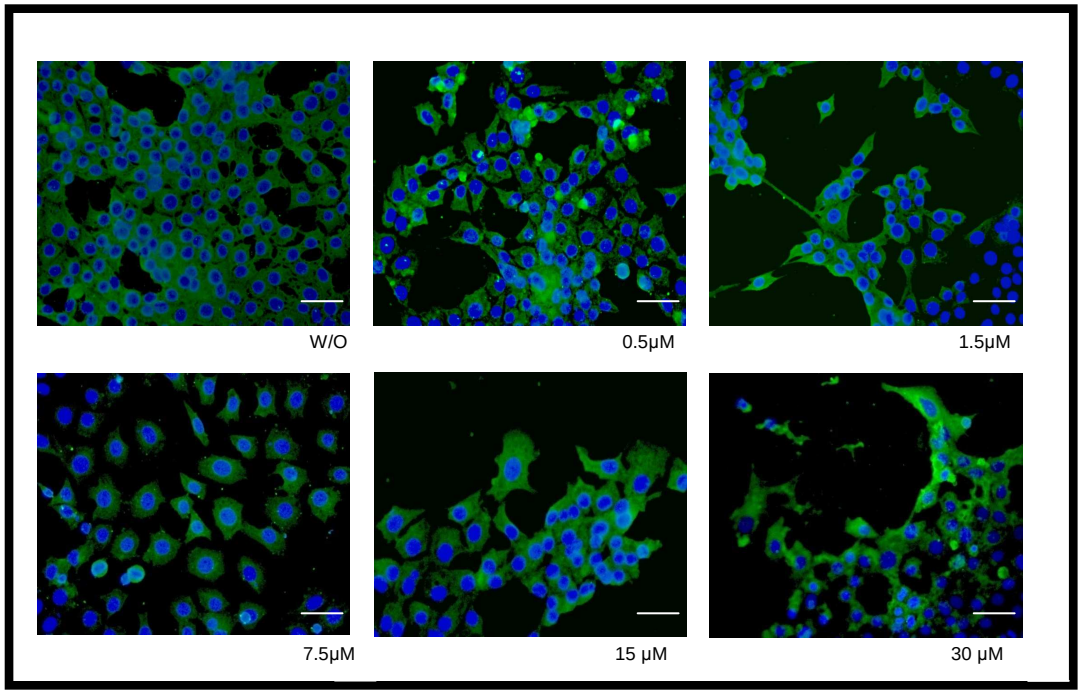

Fig.15 *Immunofluorescence staining of perilipin A/C in R2C cells treated with different CLA concentrations for 48 hours. Untreated cells immunostained for perilipin A/C (w/o). Bar = 40 µm.*

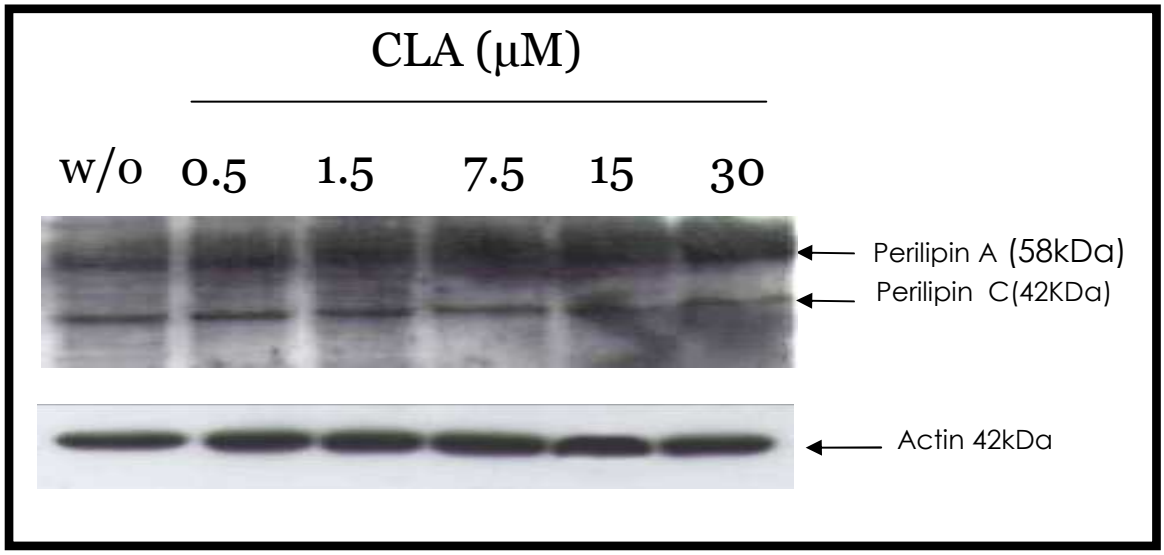

Fig.16 *Western blotting analysis of perilipin A/C in R2C cells treated with different CLA concentrations for 48 hours The Western Blotting analysis showed the expression levels of the perilipin A and perilipin C and its phosphorylation state after 48h.*

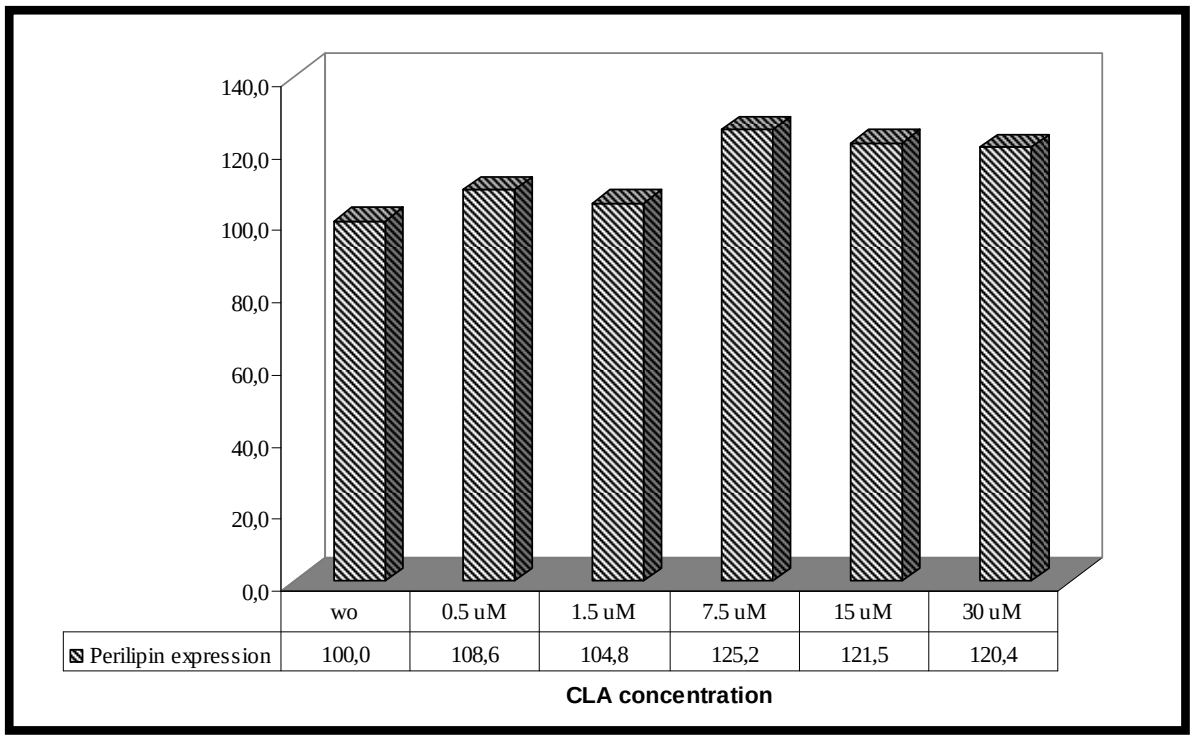

Fig.17 *Cell Based ELISA shows the expression levels of Perilipin A/C after 48h.*

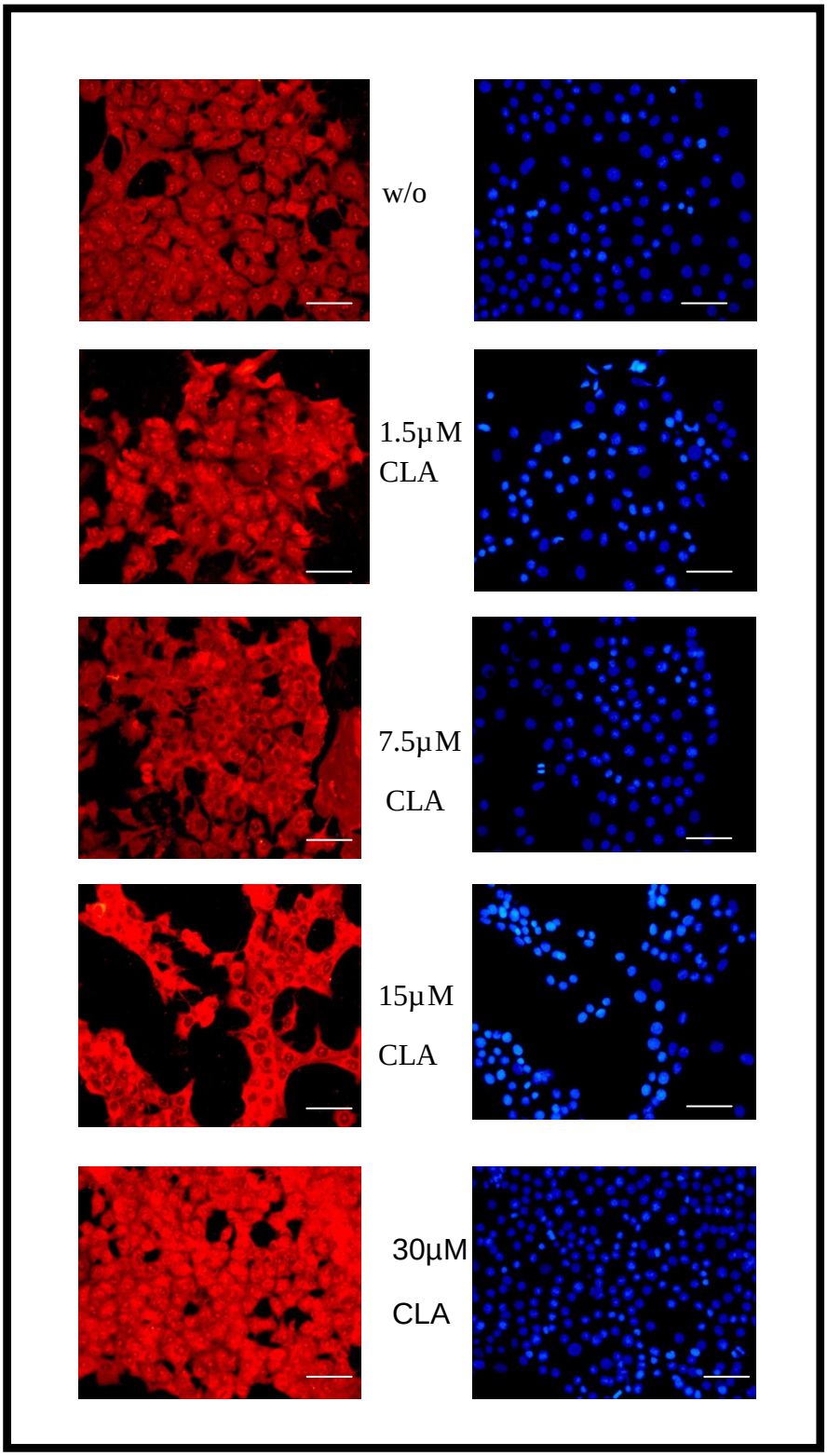

Fig.18 *Immunofluorescence of HSL in R2C cells treated with different CLA concentrations for 48 hours. Untreated cells immunostained for HSL (w/o). Bar= 40 µm*

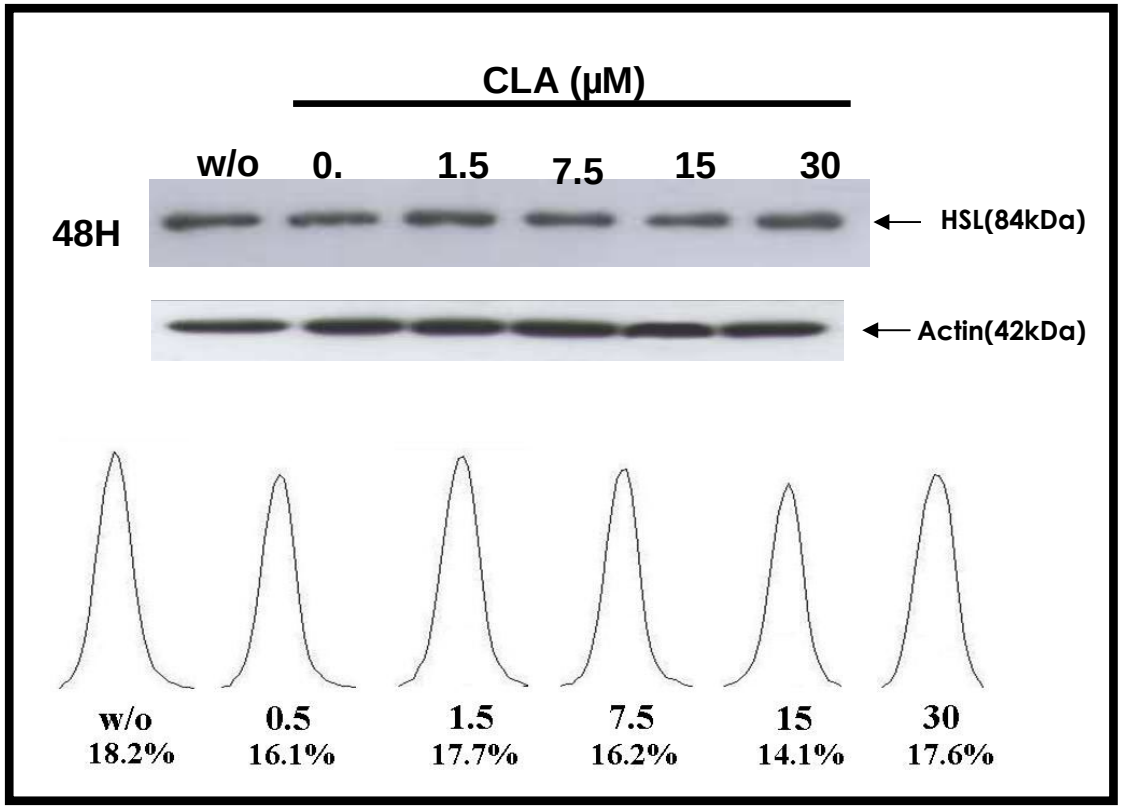

Fig.19 *Western Blotting detection of HSL in R2C cells treated with different CLA concentrations for 48 hours. Western Blotting analysis showed the expression levels of the HSL after 48h. Actin was detected in the same nitrocellulose filter after stripping as a control. Intensities of the bands of the HSL expression were calculated using the ImageJ software (NIH).*

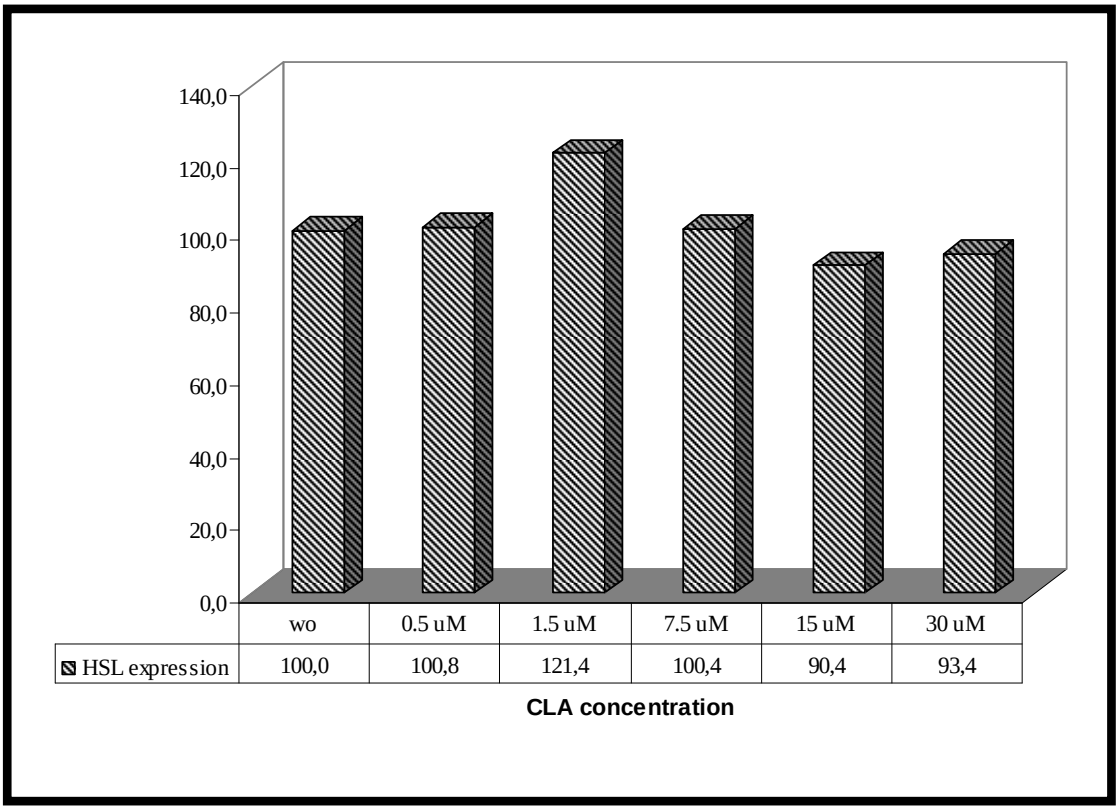

Fig. 20 *Cell Based ELISA shows the expression levels of HSL after 48h*

5. Discussion and conclusion

The possible positive effect of CLA supplementation on the body composition of sedentary or physically active subjects has been previously reported *(Campbell et al. 2008)*; however, our study is the first that provides a comprehensive set of laboratory data *in vivo* and *in vitro* and may yield some insight into the possible mechanisms induced by exercise training and CLA in the modulation of testosterone levels in the blood. Considering the lipolytic effect of the CLA mediated by an increase in the level of the perilipin A protein, and taking into account the role of perilipins and HSL in the pathways leading to steroid hormone synthesis, we investigated the effect of this supplementation *in vivo* on the levels of testosterone in the blood of physically active subjects after a bout of resistance exercise and *in vitro* on the expression and the phosphorylation state of the perilipin and HSL proteins in a tumour cell line derived from Leydig cells.

The CLA supplementation increased the blood level of total testosterone after the resistance exercise bouts, but it did not affect the estradiol, cortisol and SHBG blood levels in either Pre or Post resistance exercise bouts. The 21 days of CLA supplementation did not induce significant changes in total body weight and body composition. We suppose that despite the higher levels of testosterone that were recorded after the resistance exercise in the subjects supplemented with CLA, the supplementation period was too short to observe the hypothetical anabolic and lipolitic effect of CLA. The effects of CLA supplementation on the body composition of trained subjects, as reported in literature, are controversial. Pinkoski et al. *(Pinkoski et al. 2006)* observed a relatively small increase of lean body mass and a decrease of body fat mass in 76 moderately physically active subjects fed with

CLA for 7 weeks, while Kreider et al. *(Kreider et al. 2002)* showed that 4 weeks of CLA supplementation had no effects on the body composition of 23 bodybuilders. Our results and the results in literature exclusively on trained subjects suggest that CLA should have a significant positive effect on lean body mass and decrease body fat only if the length of supplementation is sufficiently long and the subjects do not have a very low body fat percentage, as seen in bodybuilders.

In light of this rise in testosterone blood level after resistance exercise in humans supplemented with CLA, we decided to investigate if CLA supplementation may directly influence testosterone secretion *in vitro*. For this purpose we used a leydig tumour (R2C) cell line, which is steroidogenic without cAMP stimulation *(Servetnick et al.1995)*. The choice to investigate the effect of CLA on a rat tumour cell line was made because: i) it is un-ethical to perform a testicular biopsy in humans without any prior results from animal studies that justify it, ii) it is impossible to obtain a 100% pure Leydig cell culture from animal testes and these cells are hard to maintain in culture, unlike the tumour cell line and iii) only the R2C cell at a basal conditioning level produce steroid hormones, simulating the activation of these cells in humans after a resistance exercise bout, while Leydig cells isolated from animal testis require the addition of human chorionic gonadotropin to produce steroid hormones.

As seen in human experiments, CLA also induced an increase in testosterone production in *in vitr*o experiments. Even if there is a basal increase in the testosterone release in the culture medium due to the longer time in culture, point by point increases may be appreciated.

The mechanism by which CLA may induce an increase in testosterone level in blood and in culture media is not known. In normal leydig cells, testosterone production is dependent upon cholesteryl esterase activation by cAMP *(Servetnick et al. 1995)* and

lipid hydrolysis depends upon dislocation of phosphorylated perilipin A/C proteins from the membrane of the lipid droplet *(Brasaemle et al. 2009)*. The HSL protein, whose activation depends from the phosphorylation of perilipin, is know free to hydrolyse cholesteryl esters. The role of perilipin and HSL in testosterone synthesis is poorly understood, but it should represent only one of the complex steps of the testosterone synthesis pathway. Hence we decided to investigate the protein levels and the phosphorylation state of perilipins A/C and HSL in R2C cells, which are easily cultured and are routinely used in the study of testosterone synthesis *(Sirianni et al. 2009).* Perilipin C isoform seems not to be involved in the CLA treatment effect. Considering that the cAMP signalling pathway is inhibited in these cells *(Servetnick et al. 1995)* and also that testosterone is increased following CLA treatment, we can assume that CLA may induce the phosphorylation of the perilipin proteins in a cAMP independent manner; therefore, we hypothesise that CLA may have an effect on the steps by which cholesteryl esterase leads to testosterone synthesis in the cytoplasm.

In conclusion, considering both the *in vivo* and *in vitro* results, we have demonstrated that CLA supplementation induces an increase in the testosterone levels via the Leydig cells of the testis, and that this effect is independent on perilipin phosphorylation and HSL expression; however, the mechanism by which CLA increases the testosterone levels remains to be established.

In the next future we would like to evaluate the expression and activation of other proteins known to be involved in the pathway of the synthesis of testosterone.

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