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School of Medicine

Graduate Program “Molecular Basis of Human Diseases”

# Immunological and genetic markers in obesity and aging

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Master Thesis

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# Ανοσολογικοί και γενετικοί δείκτες στην παχυσαρκία και τη γήρανση

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Μεταπτυχιακή Διπλωματική Εργασία

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## Abstract

The present master thesis aims to the study of inflammatory factors and genetic markers in obesity and aging. Obesity is a health problem that affects millions of people annualy and it is associated with inflammation and many comorbidities. Aging is a process of progressive reduction in cells' function leading to a variety of diseases. Blood samples were collected from 39 obese volunteers and 27 lean volunteers. PBMCs were isolated by density gradient centrifugation and leukocyte subpopulations were assessed by flow cytometry. Serum was isolated by centrifugation, while cells were cultured for 48h either with B or BL (with LPS) medium. From 66 particpants, 60 samples serums and 30 samples of cells that were cultured in B or BL (LPS induction) medium (from 15 individuals) were used for IL-6 ELISA. Genotyping for different SNPs was held in order to measure genetic risk scores for obesity and telomere length. Statistical analysis was performed using the unpaired Mann-Whitney t-test (two- tailed), multiple t-test, correlation co-efficience, one-way ANOVA test and Sperman's correlation. Data demonstrate that obesity is associated with a significant increase in the majority of white blood cells, meaning circulating PBMCs, neutrophils, monocytes and lymphocytes. As far as lymphocytes are concerned, $CD3^+$ T cells, $CD8^+$ T cells, T regulatory cells and $CD4^+$ T cells, where naïve, TEMRA, effector and central memory T cells belong, as well as B and NK cells are elevated in obese participants confirming the proinflammatory status of obese individuals. Conversely, reduction in basophils, eosinophils and non-classical monocytes is reported in the obese group. Furthermore, it was found that IL-6 is strong correlated with BMI, physical activity and alcohol consumption can slightly increase IL-6 levels, while smoking and coffee consumption are linked to small reduction of IL-6 concentrations. IL-6 levels are notably elevated in samples that were induced by LPS. Genetic risk score for obesity is positively correlated with BMI as it is a marker of predisposition to obesity, whereas genetic risk score for telomere length that reveals cardiological age is associated with increased levels of IL-6. So, inflammation plays important role in obesity and aging, while BMI is a significant factor for a person's health.

**Key Words:** obesity, inflammation, aging, IL-6, PBMCs, lymphocytes, BMI, genetic risk score, telomere length

## Περίληψη

Η παρούσα μεταπτυχιακή εργασία στοχεύει στη μελέτη φλεγμονωδών παραγόντων και γενετικών δεικτών στην παχυσαρκία και τη γήρανση. Η παχυσαρκία είναι ένα πρόβλημα υγείας που πλήττει εκατομμύρια ανθρώπους ετησίως και σχετίζεται με τη φλεγμονή και πολλές συννοσηρότητες. Η γήρανση είναι μια διαδικασία προοδευτικής μείωσης της λειτουργίας των κυττάρων που οδηγεί σε ποικιλία ασθενειών. Δείγματα αίματος συλλέχτηκαν από 39 παχύσαρκους και 27 νορμοβαρείς εθελοντές. Τα μονοπύρηνα κύτταρα περιφερικού αίματος (PBMCs) απομονώθηκαν με φυγοκέντρηση διαβάθμισης πυκνότητας και οι υποπληθυσμοί λευκοκυττάρων αναλύθηκαν με κυτταρομετρία ροής. Ο ορός απομονώθηκε με φυγοκέντρηση, ενώ τα κύτταρά του καλλιεργήθηκαν για 48 ώρες είτε με Β καλλιεργητικό μέσο είτε με BL (LPS-λιποπολυσακχαρίτης). Από τους 66 συμμετέχοντες, 60 δείγματα ορών και 30 δείγματα κυττάρων που καλλιεργήθηκαν σε θρεπτικό μέσο Β ή BL χρησιμοποιήθηκαν για IL-6 ELISA. Διεξήχθη γονοτύπηση για διαφορετικά SNPs-πολυμορφισμούς με σκοπό τη μέτρηση των γενετικών σκορ κινδύνου για την παχυσαρκία και το μήκος τελομερών. Η στατιστική ανάλυση πραγματοποιήθηκε χρησιμοποιώντας το Mann-Whitney t-test, το πολλαπλό t-test, το συντελεστή συσχέτισης, τη μονόδρομη δοκιμή ANOVA και τη συσχέτιση Sperman. Τα στοιχεία δείχνουν ότι η παχυσαρκία σχετίζεται με σημαντική αύξηση στην πλειονότητα των λευκών αιμοσφαιρίων, δηλαδή των κυκλοφορούντων PBMCs, ουδετερόφιλων, μονοκυττάρων και λεμφοκυττάρων. Όσον αφορά τα λεμφοκύτταρα, τα $CD3^+$, $CD8^+$ Τ κύτταρα, τα Τ ρυθμιστικά κύτταρα και τα $CD4^+$ Τ κύτταρα, όπου ανήκουν τα naïve, TEMRA, κεντρικής μνήμης και τελεστές μνήμης Τ κύτταρα, καθώς και τα Β και NK κύτταρα αυξάνονται σε παχύσαρκους συμμετέχοντες επιβεβαιώνοντας την προφλεγμονώδη κατάσταση των παχύσαρκων ατόμων. Αντίθετα, η μείωση των βασεόφιλων, ηωσινόφιλων και των μη-κλασσικών μονοκυττάρων παρατηρείται στην παχύσαρκη ομάδα. Επιπλέον, βρέθηκε ότι η ιντερλευκίνη 6 (IL-6) συσχετίζεται ισχυρά με το ΔΜΣ, η σωματική δραστηριότητα και η κατανάλωση αλκοόλ μπορεί να αυξήσει ελαφρώς τα επίπεδα της IL-6, ενώ το κάπνισμα και η κατανάλωση καφέ συνδέονται με μικρή μείωση των συγκεντρώσεων της IL-6. Τα επίπεδά της είναι ιδιαίτερα αυξημένα σε δείγματα κυττάρων που επήχθησαν με LPS. Τα γενετικά σκορ κινδύνου για την παχυσαρκία συσχετίζεται με θετικά με το ΔΜΣ, καθώς αποτελεί δείκτη προδιάθεσης για την παχυσαρκία, ενώ το γενετικό σκορ κινδύνου για το μήκος

των τελομερών που αποκαλύπτει την καρδιολογική ηλικία, σχετίζεται με αυξημένα επίπεδα της IL-6. Επομένως, η φλεγμονή παίζει σημαντικό ρόλο στην παχυσαρκία και τη γήρανση, ενώ ο ΔΜΣ είναι ένας σημαντικός παράγοντας για την υγεία ενός ατόμου.

**Λέξεις-Κλειδιά:** παχυσαρκία, φλεγμονή, γήρανση, ιντερλευκίνη-6, Μονοπύρηνα κύτταρα περιφερικού αίματος, λεμφοκύτταρα, ΔΜΣ (Δείκτης Μάζας Σώματος), γενετικό σκορ κινδύνου, μήκος τελομερών

# Contents

# 1. Introduction

## 1.1. Obesity

Obesity is a very common health problem that occurs both in the developed and developing countries. It does not only related to body fat and its possible negative external appearance, but it mainly concerns the health challenges that it causes (Kopelman, 2000). Based on the World Health Organization (WHO), individuals with a body mass index (BMI) 18-25 $kg/m^2$ are considered as normal or lean, people with BMI 25-30 $kg/m^2$ are considered as overweight, those with a BMI 30-40 $kg/m^2$ are considered as obese and a BMI>40 $kg/m^2$ is considered morbid obesity [1]. More specifically, overweight and obese people are those that are characterized by an excessive or abnormal fat accumulation in whole body and its cells, which is associated with many health problems causing the death of approximately 4 million of people annually [1]. Overweight are approximately 30-70% of adults and obese adults hold 10-30% in Europe (Flegal, et al., 2010), whereas in the United States, obesity is prevalent in 34.9% of adults (Ogden, et al., 2014).

Obesity is thought by many researchers as a metabolic disease that can cause chronic inflammation (Suganami, et al., 2012), whereas it is responsible for many comorbidities, such as cardiovascular diseases, hypertension, diabetes mellitus type 2 (T2DM) and some types of cancer (Hubert, et al., 1983; Calle, et al., 2003). It is well known that obesity is among the top 10 causes of mortality worldwide (Mitchell, & Shaw, 2015). Malnutrition and poverty are mainly the causes of obesity in developing countries, such as Africa (Gerriets, & MacIver, 2014), while many factors, such as genetic and environmental are the causes of obesity (Kilic, et al., 2014) **(Figure 1)**. Generally, it is caused due to the accumulation of lipids in many tissues throughout the body and not only in the adipose tissues due to the imbalance between the food intake and the energy expenditure (Suganami, et al., 2012). This imbalance can lead to increased number (hyperplasia) and size (hypertrophy) of adipocytes (Gregor, & Hotamisligil, 2011).

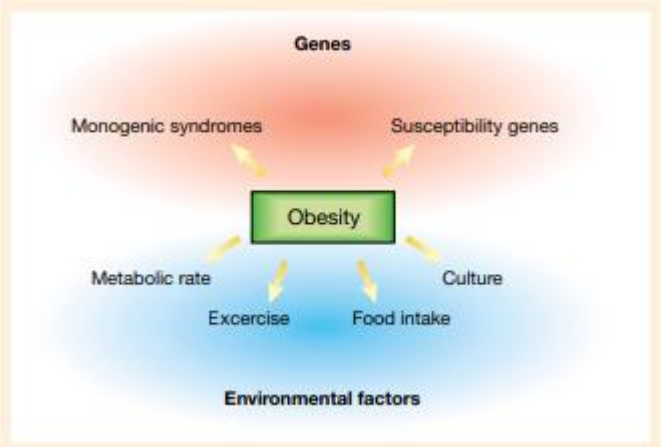


***Figure 1*** *Factors that can cause obesity (Kopelman, 2000).*

Adipose tissue is the part of the body where lipids are stored in order to be accessible for use mainly at conditions of lacking of energy (Mayoral, et al., 2015). The size of adipose tissues grows when food intake is more than is needed leading to insulin resistance (Majoral, et al., 2015). When the size of adipocytes is huge, the excess lipids, that cannot be stored in the cells, are stored in the liver causing dyslipidemia, increase in the levels of plasma free fatty acids, low- density lipoprotein (LDL) and triglycerides, and decrease in high- density lipoprotein (HDL) levels.

In obesity, white adipose tissues are characterized by chronic inflammation that can lead both to insulin resistance in pancreas and muscles and to a decrease in adiponectin secretion (Yamagata, & Yoshizawa, 2018). Adipokine production, meaning the cytokines that are produced by adipocytes, including leptin and adiponectin, contributes to maintenance of inflammation, which can lead to many pathological processes (Lago, et al., 2007). Also, in this inflammation many macrophages are produced that secrete different cytokines, such as TNF-a, which can induce insulin resistance and lipolysis (Hotamisligil, et al., 1994; Zhang, et al., 2002), IL-6 and TGF-β1 (Fried, et al., 1998; Weisberg, et al., 2003). White adipose tissues are converted to brown ones for the prevention of weight gain and obesity as they produce heat and can expend energy (thermogenesis) through exercise and decreased food consumption (Cypess, et al., 2009).

Adipocytes can change the cytokines that are produced during obesity causing the so-called low-level chronic inflammation (Fain, 2006), where inflamed vascular endothelium and activated immune cells are involved, affected by nutrient excess, dyslipidemia, hyperglycemia or oxidative stress (Dalmas, et al., 2011). For example,

researchers have found that the levels of the anti- inflammatory cytokine interleukin-10 (IL-10) are increased during obesity (Lago, et al., 2007), while the cause of the elevated levels of M1-polarized proinflammatory macrophages and $CD8^+$ T cells that can enhance insulin resistance is the excessive energy storage in obese adipocytes (Norata, et al., 2015). Insulin resistance is when insulin is produced mainly by pancreatic b cells rather than the increased uptake of glucose by liver and muscle, leading to β-cell malfunction (Bjørndal, et al., 2011). This inflammation can cause different pathological problems in a variety of tissues, with the most significant to be the obesity-driven liver inflammation, which can lead to the development of non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH) and cirrhosis (Norata, et al., 2015). Lastly, "meta-inflammation", the inflammation in metabolic tissues, represents the absence of clinical manifestations of inflammation and a modest increase in circulating proinflammatory agents (Medzhitov, 2008).

## 1.2. Inflammation-Cytokines

Adiponectin is a cytokine that can enhance the production of anti-inflammatory cytokines, such as IL-10 (Wolf, et al., 2004; Carbone, et al., 2012), while it can inhibit the production of proinflammatory cytokines, like TNF-α and IFN-γ (Carbone, et al., 2012). However, its production is suppressed by proinflammatory cytokines including TNF-α (Maeda, et al., 2002; Carbone, et al., 2012) and IL-6 (Fasshauer, et al., 2003; Carbone, et al., 2012).

Levels of TNF-a, which production is known to organize glucose and lipid metabolism, inflammatory processes and immune response (Tanaka, et al., 2001), have been found to be elevated during obesity, while they decreased after weight loss (Kern, et al., 1995; Bruun, et al., 2002). Similarly, the levels of TNF receptor were observed to be increased at obese people (Mantzoros, et al., 1997; Tsukui, et al., 2000).

Also, levels of interleukin-8 (IL-8), C-C chemokine ligand 2 (CCL2)/monocyte chemotactant protein 1 (MCP-1), CCL5/regulated on activation (Dalmas, et al., 2011; Hotamisligil, 2006; Poitou, et al., 2005), IL-6 (Mohamed-Ali, et al., 1997) and C-reactive protein (CRP) (Visser, et al., 1999; Yudkin, et al., 1999; Visser, et al., 2001; Tchernof, et al., 2002) are increased during obesity causing inflammation,

while their levels are decreased through weight loss (Forsythe, et al., 2008). In inflammatory conditions, TNF-a enhances the production of IL-6, which induces the production of CRP in order to cause inflammation (Yudkin, et al., 1999).

### 1.2.1. Polymorphonuclear leukocytes

**Neutrophils**

Neutrophils are the most abundant category of circulating leucocytes **(Figure 4a)** (Pecht, et al., 2014) and the cells that constitute the first line of defense of human's organism against possible infections, by secreting a variety of cytokines, such as IL-1β, IL-8, TNF-α and MIP-1a **(Figures 2 & 3)** (Asghar, & Sheikh, 2017). These cells can neutralize microbia either by phagocytosis or by degranulation of their antimicrobial factors (Mraz, & Haluzik, 2014), while they can regulate and stimulate the other lines of defense for further combat of pathogens (Elgazar-Carmon, et al., 2008).

Numbers of neutrophils have been found increased during obesity (Wypych, et al., 2017) and in fact more rapid than macrophages (Nguyen, et al., 2011), while they decrease through weight loss (Pecht, et al., 2014).

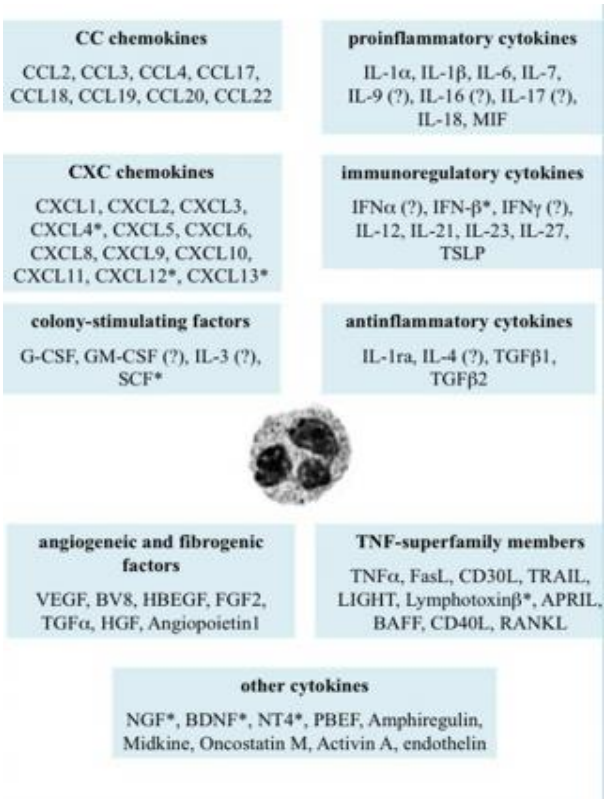


***Figure 2*** *Cytokines that can be produced or expressed by neutrophils (Tecchio, et al., 2014).*

| | |
|---|---|
| Proinflammatory cytokines | IL-1α, IL-1β, IL-6, IL-16, IL-18, MIF |
| Anti-inflammatory cytokines | IL-1ra, TGFβ1, TGFβ2 |
| Chemokines | CXCL1, CXCL2, CXCL8, CXCL9, CXCL10, CXCL11, CCL2, CCL3, CCL4, CCL17, CCL18, CCL19, CCL20,CCL22, CCL23 |
| Immunoregulatory cytokines | IL-22, IL-23 |
| TNF family members | TNF, CD30L, FasL, TRAIL, APRIL, BAFF, RANKL |
| Colony stimulating factors | G-CSF |
| Angiogenic and fibrogenic factors | VEGF, FGF2, BV8, HB-EGF, TGFα, HGF, Angiopoietin1, EphrinB1 |
| Other cytokines | Oncostatin M, Activin A, PBEF, endothelin, Midkine |

***Figure 3*** *Cytokines that neutrophils can express or produce (Cassatella, et al., 2019).*

**Eosinophils**

Eosinophils constitute 1-4% of circulating leucocytes **(Figure 4a)** (Pecht, et al., 2014) and their role in inflammation is to secrete IL-4 and IL-13 and to maintain adipose tissue macrophages in M2 state (Wu, et al., 2011). As far as obesity concerns, any relation-difference between obese people and the numbers of eosinophils has not been observed (Pecht, et al., 2014). However, the numbers of eosinophils were decreased in visceral adipose tissues of obese mice, while mice without eosinophils have insulin resistance and inflammation (Huh, et al., 2014).

**Basophils**

Basophils that are responsible for hypersensitivity inflammatory allergic reactions through binding antigen-specific immunoglobulin E (IgE), characterize the smallest group of granulocytes and circulating leukocytes **(Figure 4a)** (Pecht, et al., 2014). There was found no association between basophils and obesity as a result more research is needed (Pecht, et al. 2014).

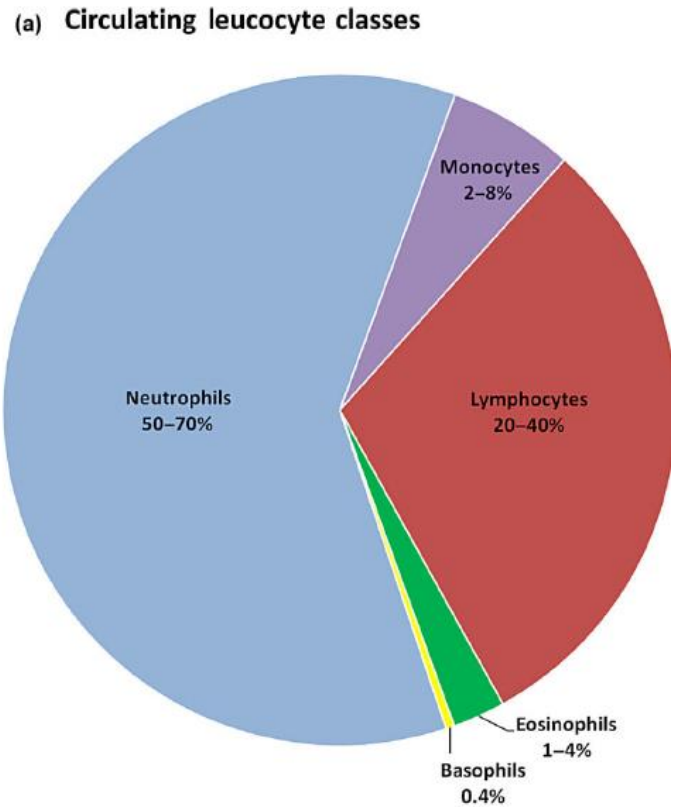


***Figure 4a*** *Percentages of circulating leucocyte classes (Pecht, et al., 2014).*

### 1.2.2. Monocytes

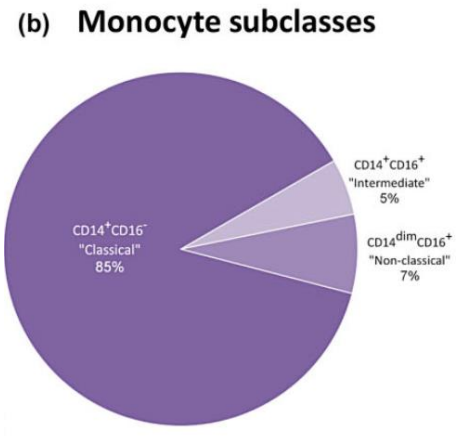


***Figure 4b*** *Percentages of subclasses of monocytes that are based on the expression of the CD14 and CD16 markers (Pecht, et al., 2014).*

Monocytes that consist 2-8% of circulating leukocytes **(Figure 4a)** (Pecht, et al., 2014) are phagocytes that derive from the bone marrow. Their role is to differentiate monocytes to tissue macrophages or myeloid lineage dendritic cells and they belong to the first lines of human's organism defense against pathogens by producing

inflammatory cytokines, phagocytosis and secreting nitric oxide, reactive oxygen species and myeloperoxidase (Cros, et al., 2010).

M1 macrophages produce elevated levels of cytokines including IL-6 and TNF-a, while M2 macrophages can produce cytokines like IL-1Ra and IL-10 (Asghar, & Sheikh, 2017). During obesity macrophages can convert from M2 to M1 state, a process that is associated with TNF-a, IFN-γ and microbial products such as lipopolysaccharides (LPs) (Asghar, & Sheikh, 2017).
Macrophages secrete cytokines, like TNF-a, IL-1, IL-6, IL-8, IL-12, IL-18, IL-23, IL-27 (proinflammatory cytokines), IL-10, TGF-b (anti-inflammatory cytokines), VEGF, CXCL1 and CXCL2 (MIP-2α), CCL5 (RANTES), CXCL8 (IL-8), CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (IP-9), MCP-1, MIP-1a (Schutte, et al., 2009; Duque, et al., 2014).

Human monocytes can be divided in three subtypes based on the expression of the markers CD14, the bacterial lipopolysaccharide (LPS) co-receptor, and CD16, the low-affinity FcγIII receptor (Pecht, et al., 2014).

$CD14^{+}CD16^{-}$ or classical monocytes are the major subset of monocytes **(Figure 4b)**. They are responsible for the expression of the chemokine receptor C-C type 2 (CCR2) and CX3CR1 at high levels, as well as CCR5 at lower levels (Poitou, et al., 2011; de Matos, et al., 2016). They produce IL-6, IL-8 and ROS in response to LPS, as well as IL-10 at high levels and TNF-α at low levels (Cros, et al., 2010). Their role is tissue repair (Gómez-Olarte, et al., 2019).

$CD14^{+}CD16^{+}$ or intermediate monocytes constitute 5% of monocyte subclasses **(Figure 4b)**. They are responsible for the expression of markers CD43, CD45RA, HLA-DR, epidermal growth factor module-containing mucinlike receptor 2 (EMR2), Ig-like transcript 4 (ILT-4) (Ziegler-Heitbrock, 2007), CD11c, CD16, CD62L, CD163 and CX3CR1. They are increased in obese adults and children especially in cases of atherosclerosis and hyperglycemia (Schipper, et al., 2012). They contribute to angiogenesis expressing MHC class II (Gómez-Olarte, et al., 2019).

$CD14^{-}CD16^{+}$ or non-classical monocytes comprise 7% of total monocytes **(Figure 4b)**. They produce CCR5, CD11b and CD163 at a lower level than intermediate monocytes (de Matos, et al., 2016), while they secrete proinflammatory factors, such as IL-1β, TNF-α and CCL3 (Cros, et al., 2010). They are responsible for the

production and secretion of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8 (Gómez-Olarte, et al., 2019).

Monocytes are more abundant during obesity (Kullo, et al., 2002; Marzullo, et al., 2014), while after weight loss their numbers are decreased producing lower levels of TNF-a (Tanaka, et al., 2001; Magrone, et al., 2017). Also, monocytes in obesity produce reactive oxygen species at high proportions (Degasperi, et al., 2009), have increased expression of the proinflammatory markers IL-6 and TNF-α and decreased expression of the anti- inflammatory IL-10 (Satoh, et al., 2010). More specifically, $CD14^{+}CD16^{+}$ and $CD14^{dim}CD16^{+}$ cells were increased in obese compared to lean participants as a result monocytes are linked to BMI, fat mass and inflammatory factors (Cottam, et al., 2002; Poitou, et al., 2011; Krinninger, et al., 2014; Rogasev, et al., 2010). $CD14^{+}CD16^{-}$ cells are usually decreased in obese compared to lean individuals (Poitou, et al., 2011; Rogasev et al., 2010).

### 1.2.3. Lymphocytes

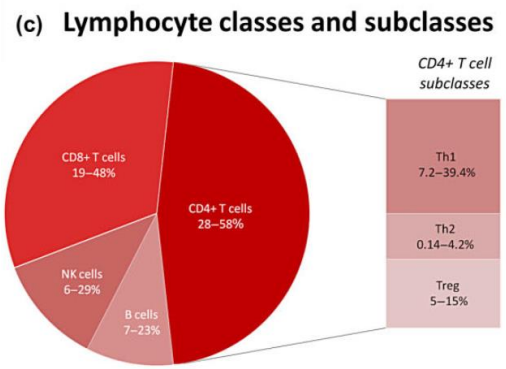


***Figure 4c*** *Percentages of classes and subclasses of lymphocytes (Pecht, et al., 2014).*

Lymphocytes constitute 20-40% of total leucocytes **(Figure 4a)** and can be categorized in three subclasses, T cells either CD4+ or CD8+ (adaptive immunity), B cells (adaptive immunity) and natural killer cells (innate immunity). During obesity, it has been observed that the numbers of lymphocyte were elevated, while they were correlated not only with BMI, but also with other characteristics, such as age, sex and smoking (Bastard, et al., 2000).

*1.2.3.1. T cells*

T cells are the most abundant subclass of lymphocytes **(Figure 4c)** and are mainly characterized by the presence of the CD3 marker, as well as after the production in bone marrow and maturation in thymus, they are responsible for the adaptive immune system (Jäger, & Kuchroo, 2010). Based on the expression of surface markers, T cells can be also subdivided in $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T cells can be categorized into T helper and T regulatory cells, while $CD8^+$ T cells are characterized as T cytotoxic cells. T helper cells or $CD4^+$ cells are further divided in several subtypes mostly based on the production of different cytokines, for instance Th1 that produce IFN-γ and TNF-α, Th2 that express IL- 4, IL- 5, IL-9, IL- 13 and IL-25, Th17 that synthesize IL- 17, IL- 21 and IL- 22 and Th22 that produce IL- 22 in the absence of IL- 17 (Asghar, & Sheikh, 2017; Touch, et al., 2017).

| $CD4^+$ Subset | Cytokines | Transcription factors | Inhibitory transcription factors |
|---|---|---|---|
| Th1 | IL12, **IFNγ** | T bet, STAT1, STAT4, Runx 3, Eomes, Hlx | GATA3 |
| Th2 | **IL4**, IL2 | GATA3, STAT6, STAT5, STAT3, Gfi-1, c-Maf, IRF4 | T-bet, Runx3 |
| Th17 | IL6, **IL 21**, IL 23, TGF-β | RORγt, STAT3, RORα, Runx1, Batf, IRF4, AHR | T-bet$^+$ Runx1, Smad3 Runx1$^+$FOXP3 |
| Tfh | IL6, IL21 | Bcl6, STAT3 | |
| iTreg | TGF-β, IL2 | FOXP3, Smad2, Smad3, STAT5, NFAT | |
| Th9 | TGF-β, IL4 | IRF4 | |
| Tr1 | IL27, IL10 | c-Maf, AhR | |

***Figure 5*** *Cytokines from CD4 T cells (Luckheeram, et al., 2012).*

It has been found that the numbers of T cells are elevated in obese compared to lean participants (Nieman, et al., 1999; Van der Weerd, et al., 2012), but these high levels were not observed in morbidly obese subjects (Ilavská, et al., 2012) and women (Al-Sufyani, & Mahassni, 2011).

1.2.3.1.1. $CD4^+$ T cells

$CD4^+$ T cells comprise 28-58% of subclasses of lymphocytes and their role in immune system is secrete cytokines and transfer the appropriate immune cells in the place of inflammation. Their number was increased in overweight (Womack, et al., 2007), obese (Nieman, et al., 1996; Scanga, et al., 1998; O'Rourke, et al., 2005; Womack, et al., 2007; Al-Sufyani, & Mahassni, 2011), morbidly obese people (Hanusch-Enserer, et al., 2011; Laurson, et al., 2011). Also, these cells are positively

correlated apart from BMI with insulin sensitivity (O'Rourke, et al., 2005; Van der Weerd, et al., 2012), while no difference has been found in the numbers after weight loss (Hanusch-Enserer, et al., 2003; Viardot, et al., 2012).

Regulatory T cells (Tregs), which are responsible for the maintenance of immune homeostasis and the production of the anti-inflammatory cytokines IL-10 and TGF-β creating an anti-inflammatory environment, also, by inducing M2 polarization via IL-4, IL-10 and IL-13 production (Tiemessen, et al., 2007), reducing M1 polarization and improving insulin sensitivity (Feuerer, et al., 2009; Deiuliis, et al., 2011), comprise 5-15% of circulating $CD4^+$ T cells **(Figure 4c)** (Matarese, et al., 2010). During obesity, the number of Treg cells is decreased in adipocytes (Esser, et al., 2013) and in T2D patients (Jagannathan-Bogdan, et al., 2011; Zeng et al., 2012) resulting in the reduction of anti-inflammatory cytokines (Feuerer, et al., 2009). However, it is observed that in morbidly obese patients Treg cell numbers are elevated (Van der Weerd, et al., 2012).

$CD4^+$ T cells can be also characterized by the expression of the leukocyte common antigen isoforms, CD45RA and CD45RO, and the expression of the homing chemokine receptor CCR7 and as a result they can be categorized into four subtypes: naïve (N, $CD45RA^+CCR7^+$), central memory (CM, $CD45RO^+CCR7^+$), effector memory (EM, $CD45RO^+CCR7^-$) and terminally differentiated effector memory cells (TEMRA, $CD45RA^+CCR7^-$). Differentiation happens between them through the stages Naïve→ CM→ EM→ TEMRA, where TEMRA do not differentiate further. Changes in the expression of CD45 isoforms can lead to a variety of diseases, including metabolic ones (Li, et al., 1989), while a decrease in $CD4^+CD45RA^+$ and an increase in $CD4^+CD45RO^+$ were observed in patients with non-alcoholic steatohepatitis (Inzaugarat, et al., 2011).

During obesity, the levels of $CD4^+CD45RO^+$ and $CD4^+CD45RA^+CCR7^+$ T cells have been found to be decreased, while the numbers of $CD4^+CD45RO^+CCR7^-$ cells were elevated (Mauro et al., 2017). After weight loss the number of memory and naïve cells were increased, while effector memory cells were decreased (Tanaka, et al., 2001). As far as patients with diabetes mellitus type 1 are concerned, increased levels of $CD4^+CD45RA^+$ and $CD4^+CCR7^+$ were observed, while $CD4^+CD45RO^+$ cells were elevated during treatment-protection against disease (Peakman, et al., 1994).

#### 1.2.3.1.2. $CD8^+$ T cells

T cytotoxic or $CD8^+$ cells play important role in immunity by secreting proinflammatory cytokines, such as TNF-α and IFN-γ (Cox, et al., 2013). $CD8^+$ T cells are suppressed by the anti-inflammatory cytokines IL-10 and TGF-β (Cox, et al., 2013). Numbers of $CD8^+$ T cells have been found to be elevated in overweight, obese and morbidly obese people compared to lean ones (O'Rourke, et al., 2005; Womack, et al., 2007), while IFN-γ is also increased in obesity (Asghar, & Sheikh, 2017). Memory and local effector $CD8^+$ T cell numbers have been found elevated in obese (Nishimura, et al., 2009). After weight loss no difference has been observed (Lynch, et al., 2009). On the other hand, insulin sensitivity is associated with a reduction in $CD8^+$ T cells levels under obese condition (Nishimura, et al., 2009).

Viruses stimulate the production of proinflammatory cytokines which can increase $CD8^+$ T cells. These cytokines can be interferons (IFNα and IFNβ), IL-12 family members, IL-6 and related cytokines IL-2, IL-21, IL7 and IL-15, as well as IL-1 family members including IL-18 and IL-33 (Cox, et al., 2013).

#### *1.2.3.2. B cells*

B lymphocytes comprise 7-26% of total lymphocytes that are circulated in the body, a proportion smaller than this of T cells **(Figure 4c)** (Pecht, et al., 2014). Generally, in B cells the cytokines that are produced are IL-7, IL-4, IL-6, IL-10, IFN-α, IFN-β, IFN-γ and the chemokine CCL28 (Vasquez, et al., 2015). Regulatory B cells (Breg) are distinguished by their ability to secrete IL-10 or TGFβ-1, while effector B cells produce cytokines such as IL-2, IL-4, TNFα, IL-6 or IFNγ, IL-12 and TNF-a (Lund, 2008).

Under overweight and obese state the numbers of B cells were increased compared to lean participants especially in women (Ilavská, et al., 2012; Phillips, et al., 2010). Also, these elevated levels of B cells are associated with increased risk for metabolic syndrome, insulin resistance and obesity (Phillips, et al., 2010). During obesity, B cells can produce more proinflammatory (increased secretion of IL-8, IFN-γ and IL-17) and less anti- inflammatory (low production of IL-10) cytokines (Frasca, et al., 2016).

### 1.2.4. Natural killer (NK) cells

Natural killer (NK) cells that belong to $CD3^-$ lymphocytes constitute 10% of peripheral blood mononuclear cells (PBMCs) (Moretta, et al., 2002) and 6-29% of human lymphocytes **(Figure 4c)** (Pecht, et al., 2014). NK cells in the liver comprise 30% of total lymphocytes (Marquardt, et al., 2015), while in uterus the proportional percentage is 45% (Ivarsson, et al., 2017). NK cells participate both on innate and adaptive immunity where for the latter they produce different cytokines and chemokines, such as TNF-α and IFN-γ causing insulin resistance and induction of macrophages (Andoniou, et al., 2008).

Generally, NK cells are characterized by the secretion of immunoregulatory cytokines such as IFN-γ, different members of the IL-12 family of cytokines such as IL-12, IL-23, and IL-27 and cytokines such as IL-15 and IL-18 (Zwirner, et al., 2017). Also, by the release of cytokines, including interleukin-1 (IL-1), IL-10, IL-5, IL-13, the growth factor GM-CSF, and the chemokines MIP-1a, MIP-1, IL-8, and RANTES (Fauriat, et al., 2009) **(Figure 6)**. The stimulation of IL-12 expression and suppression of IL-15 expression in NK cells are regulated by leptin (Zhao, et al., 2003).

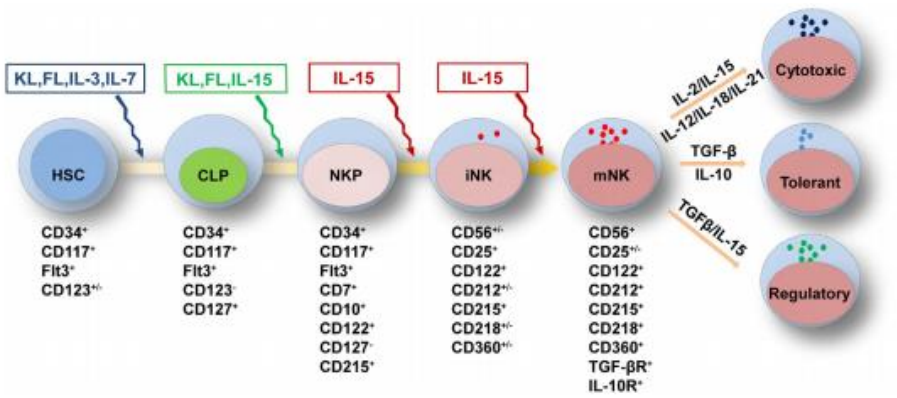


***Figure 6*** *Cytokines needed for Natural Killer (NK) cell development and function (Wu, et al., 2017).*

As far as obesity is concerned, NK cells produced higher levels of IFN-γ in lean individuals rather than obese ones (Laue, et al., 2015). Also, NK cells were decreased in the circulation of obese individuals compared to lean controls (Lynch, et al., 2009; O'Shea, et al., 2010). However, no change has been observed in weight loss (Scanga, et al., 1998).

NK cells can be categorized into three subtypes based on the expression of CD56 and CD16 markers: $CD56^{bright}CD16^-$, $CD56^{dim}CD16^-$, and $CD56^+CD16^+$. CD56 cells are

expressed on the surface of glia, neurons, skeletal muscle and NK cells and are responsible for the migration of cells to areas of infection or inflammation and in cell-cell adhesion. CD16 molecules are expressed on various cells, including monocytes, macrophages, neutrophils, NK cells and have cytotoxic function (Mousavi, et al., 2010). $CD56^{+}CD16^{+}$ cells are the most abundant subpopulation of NK cells in peripheral blood with cytotoxic responses (Moretta, et al., 2006; De Maria, et al., 2011). On the other hand, $CD56^{+/-}CD16^{-}$ cells have a significant immunoregulatory role mainly in lymphoid tissues through secretion of anti-inflammatory cytokines and regulation of T cell proliferation (Schepis, et al., 2009; Fu, et al., 2013; Morandi, et al., 2015). Researhers have found that the numbers of $CD56^{+}CD16^{+}$ NK cells in obese are higher than in lean individuals (Magrone, et al., 2017). However, no difference was observed in $CD3^{-}CD56^{+}CD16^{+}$ cells between obese and lean women (Nieman, et al., 1996).

## 1.3. Aging

Life expectancy in developing countries is about 85 years for women and 80 years for men (Sarkozy, & Coiffier, 2013). Aging is nowadays a fundamental subject as it affects people at multidimensional levels. It is concerned either with health problems and mortality or with facial appearance's problems that could cause various psychological issues. Aging is a process that affects the whole body, all the cells, tissues and organs. It is characterized by the progressive reduction of the function of the cells (Roth, et al., 2013). Aging is caused by the accumulation of a variety of mutations throughout life or due to the ability of differentiation (Vijg, & Wei, 1995) and it depends on genetic and environmental factors (Zhang, et al., 2007). As it is well known, aging can lead to death because of the fact that the changes that occur over the years can create a vulnerable place for many diseases, such as cancer, metabolic diseases (diabetes), cardiovascular and neurodegenerative diseases (Ershler, & Longo, 1997a; Lopez-Otin, et al., 2013).

Aging can be caused by DNA Damage Response (DDR), a process that happens because of free radicals, which are created from different metabolic processes (Ershler & Longo, 1997a). Reactive oxygen species and free radicals are due to oxidative stress (Irminger-Finger, 2007). These molecules can activate the transcription of specific genes that are related to inflammation and DNA damages (Irminger-Finger,

2007). When free radicals increase with age or the defense mechanisms bypass, free radical damage is accumulated (Ershler & Longo, 1997a) resulting in altered DNA and protein function leading to cell transformation and possible carcinogenesis and which can be reversed by repairing DNA damages (Anisimov, 2001).

Hallmarks of aging based on Lopez-Otin, et al., (2013) are genome instability, telomere damage, epigenetic changes, loss of proteostasis, deregulated nutrient detection, mitochondrial dysfunction, cellular senescence, stem cell depletion and altered intracellular communication (Lopez-Otin, et al., 2013). Markers of aging could be hair loss, skin ulcers, brain atrophy, cataracts, hearing problems, emotional disturbances, learning and memory deficits, abnormal circadian rhythms and increased production of reactive oxygen species (Anisimov, 2001).

For example, skin could seem to be aged due to problems in the repair mechanisms that can lead to the non-protection of skin or its damage-wrinkling, mainly, because of environmental factors and bad habits, such as UV, smoking and low body mass index (BMI) (Rexbye, et al., 2006; Christensen, et al., 2009; Roberts, et al., 2020). More specifically, skin aging happens due to various factors either intrinsic that can lead to loss of dermal cells and matrix resulting to its flattening or extrinsic, which can cause dermal elastosis, meaning accumulation of amorphous matrix of abnormal elastin (Yaar, et al., 2002). All these can lead to skin aging including wrinkles, pigmented spots, atrophic features, like telangiectasia (Yaar, et al., 2002).

Telomeres are specific nucleoprotein complexes, meaning repititive, non-coding DNA sequences (TTAGGG) that are located at the end of each chromosome (Kitagawa, et al., 2005). This nucleoprotein complex is called selterin and it is responsible for the reduction of telomere length without depending on telomerase (Lopez-Otin, et al., 2013). More specifically, no or decreased function of selterin can lead to aging (Lopez-Otin et al., 2013). Telomerase is an enzyme that its role is to prevent the development of diseases that are associated with the tissue's regenerative capacity (Lopez-Otin et al., 2013). When the cells are aged, telomerase's function is reduced resulting in smaller telomere length (Ershler, & Longo, 1997a). Telomere length progressively reduces because of the inability of DNA polymerase to replicate the 3' end of the DNA strand. For instance, lymphocytes from aged people have smaller telomere length (Ershler, & Longo, 1997b). So, the reduction of telomere length is associated with life expectancy (Ershler, & Longo, 1997b). Telomere length in the

end of chromosomes is gradually reduced after each cell cycle division (Irminger-Finger, 2007) leading to an irreversible growth arrest, cellular senescence and subsequently cell death (Anisimov, 2001). Consequently, telomere length is reduced with age and it depends on epigenetic changes (Fraga et al., 2007).

The majority of human cells do not express telomerase as a result their telomeres are gradually reduced in the end of chromosomes (Lopez-Otin et al., 2013). This is reversed in cancer cells where telomerase is expressed and telomere length is stabilized leading to immortality (Geserick, & Blasco, 2006). The expression of the human telomerase catalytic subunit, the human telomerase reverse transcriptase (hTERT), extends cells' lifespan without causing neoplastic transformation (Anisimov, 2001).

## 1.4. Aging and obesity/inflammation

Obesity and aging have common characteristics, such as genomic integrity, mitochondrial dysfunction, and cause problems in immune system, oxidative stress and inflammation (Tam, et al., 2019) **(Figure 7)**. In aging chronic, low-grade, sterile inflammation that is called as inflamm-aging is associated with many age-related diseases (Franceschi, et al., 2018). It is known that inflammatory response is strong enough at younger ages creating a balance between production and secretion of pro- and anti-inflammatory cytokines. However, over the years this response decreases, and pro-inflammatory cytokines are produced more than anti-inflammatory ones (Franceschi, et al., 2018).

Obesity and inflammation are also linked to each other. For example, obesity decreases the production of naïve T cells (Tam, et al., 2019). TNF-a and IL-6 are both markers for aging and obesity. In other words, their levels have been found to be elevated during obesity, decreased after weight loss resulting in a relation between inflammation and obesity (Ryan, & Nicklas, 2004; Deleidi, et al., 2015). Also, the presence of brown adipose tissues is reduced during aging and obesity (Jura, & Kozak, 2016). Adiponectin production and secretion is inhibited during obesity and aging, where there are high levels of endogenous adiponectin in centenarians (Jura, & Kozak, 2016).

Obesity based on studies decreases life expectancy for 5,8 years less in men and 7,1 years less in women when the age of 40 years old passes (Tam, et al., 2019). This reduction in life expectancy is due to the fact that obesity is one of the causes of the more instant aging (Tam, et al., 2019).

Obesity is also, related to telomere length. Studies have shown that telomere length not only in leucocytes, but also in adipose tissues, is negatively associated with BMI, systolic blood pressure and triglycerides (Moreno-Navarette, et al., 2010). Especially, telomere length in leukocytes is related to both obesity and smoking (Moreno-Navarette, et al., 2010). In other words, telomeres were lower in obese patients rather than lean ones and also formerly obese patients had shorter telomere length than never-obese ones (Moreno-Navarette, et al., 2010; Tam, et al., 2019). This shortening of telomere length does not affect only adipose tissues, but also skeletal muscles (Tam, e al., 2019). It has been shown that this could be reversed by physical exercise as it protects patients from telomere shortening (Zou, et al., 2017).

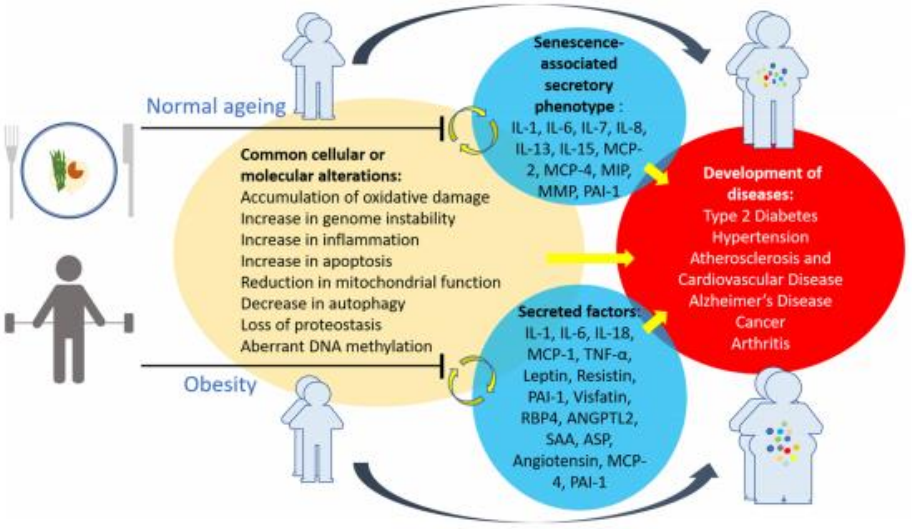


***Figure 7*** *Mechanisms of aging and obesity that can lead to the development of diseases (Tam, et al., 2019).*

## 1.5. Interleukin 6 (IL-6)

Interleukin 6 (IL-6) is mainly a pro-inflammatory cytokine that is produced by a variety of cells, including adipocytes and it acts on many tissues and organs like liver (Wang, & He, 2018). It is a single polypeptide chain of 850 amino acids (Somers, et al., 1997) with its serum concentration to range from 1-9 pg/ml for lean and obese

people (average 3–4 pg/ml) (Eder, et al., 2009). Its role is to stimulate the differentiation of T cells (such as Th17 cells) and monocytes, while it is responsible for the production of acute plase proteins (Wang, & He, 2018). It is IL-6 cytokine family member, which includes IL-11, IL-27, IL-31 (Wang, & He, 2018). It has been found to be elevated in overweight and obese people compared to lean ones (Festa, et al., 2001; Bullo, et al., 2003; Park, et al., 2005), meaning that there is a positive correlation between IL-6 and adipose tissues/BMI/insulin resistance as macrophages in adipose tissues switch from the anti-inflammatory M2 state to M1 (Pal, et al., 2014). Another mechanism could be the activation of STAT3 in T cells and inactivation of T regs that can lead to inflammation and insulin resistance (Pal, et al., 2014). Based on studies there is more statistically significant difference between IL-6 and obesity in women rather than in men, while IL-6 levels were found to be higher in obese patients with T2DM compared to obese-non diabetic ones (Popko, et al., 2010). Also, hypoglycemia or impaired glucose tolerance can lead to higher levels of IL-6 (Eder, et al., 2009) **(Figure 8)**. Mice that are deficient in IL-6 have obesity and insulin resistance (Wallenius, et al., 2002). However, between endogenous IL-6 and obesity there is a negative correlation as high levels of it is linked to a protection from obesity (Wallenius, et al., 2003). So, in this condition IL-6 has anti-inflammatory properties. Furthermore, in a study with Mexican participants a negative correlation between BMI, fasting blood glucose and triglycerides and IL-6 serum levels was found (Mendez-Garcia, et al., 2020).

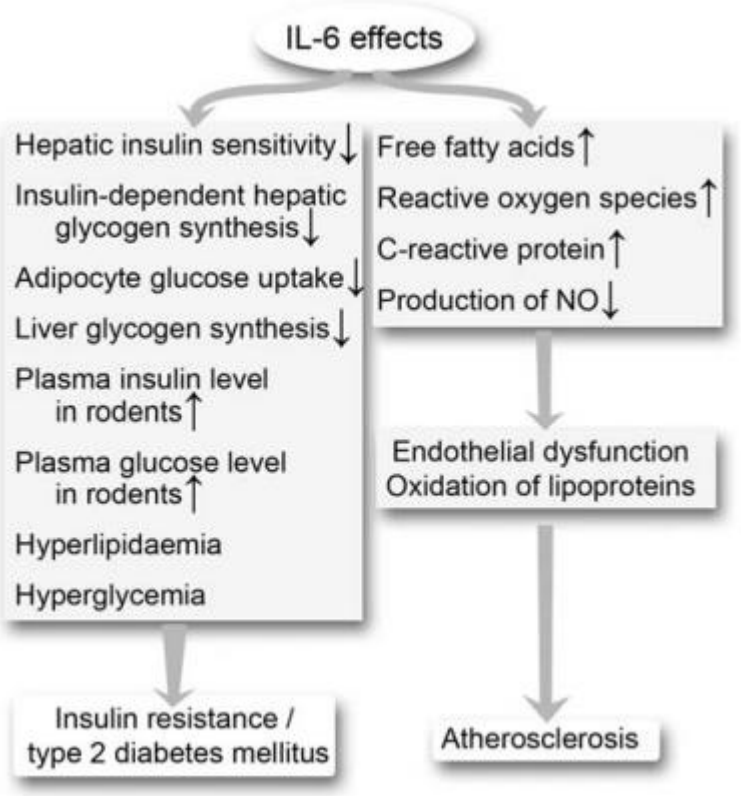


***Figure 8*** *Effects of IL-6 in insulin resistance and atherosclerosis (Eder, et al., 2009).*

So, further research is needed based on other characteristics of each population, such as genetic background, diet and physical exercise. For example, IL-6 polymorphisms rs1800795 and rs1800797 reduced the risk of obesity (Gholami, et al., 2019). As far as physical exercise is concerned, IL-6 is the cytokine that is secreted first during physical activity (Eder, et al., 2009). As a result IL-6 has been found to be increased in people that exercise, but its role is still unclear (Eder et al., 2009). Therefore, IL-6 is considered as a myokine, meaning that it is a protein produced and secreted by skeletal muscles in order to accomplish paracrine or endocrine roles in the insulin-sensitizing effects that are caused by exercise (Pal, et al., 2014). A study has shown that skeletal muscles during physical exercise can secrete IL-6 resulting in elevated insulin sensitivity and activation of glycagon-like peptide-1 (GLP-1) from pancreatic and intestinal cells which role is to secrete high levels of insulin (Pal, et al., 2014) **(Figure 9)**. Consequently, healthy diet and physical exercise can stimulate anti-inflammatory cytokines in adipose tissue, which size will be small and will be characterized by anti-inflammatory immune cells, such as M2 macrophages and Treg cells. On the other hand, the absence of health diet and physical activity can result in accumulation of body fat and inflammation by M1 macrophages and T cells leading

to insulin resistance, tumor growth, neurodegeneration and atherosclerosis (Gleeson, et al., 2011) **(Figure 10)**.

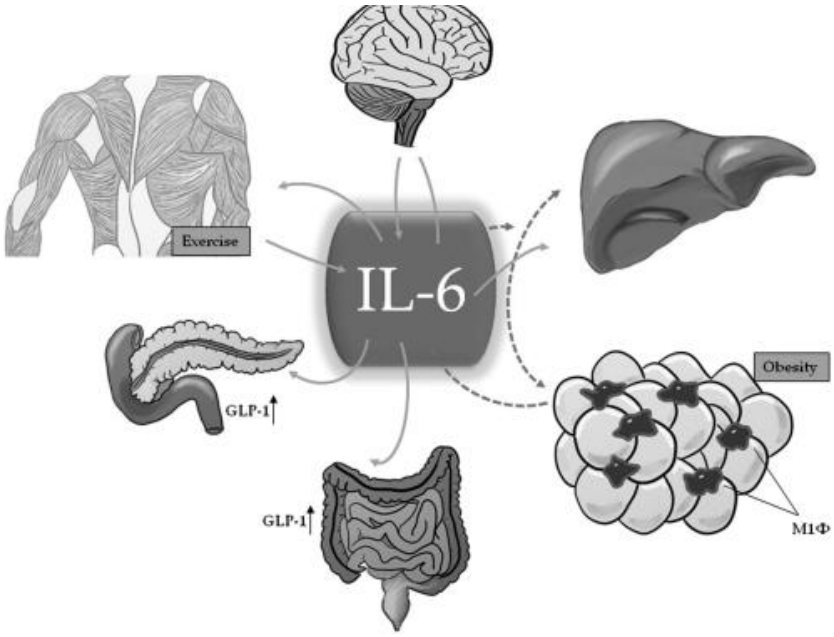


***Figure 9*** *Different applications of IL-6 in metabolism and immunology (Pal, et al., 2014).*

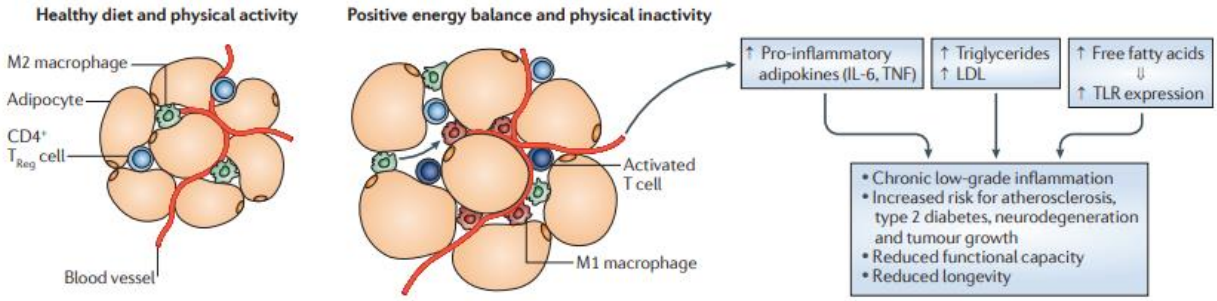


***Figure 10*** *Diet and physical exercise and its possible results in inflammation (Gleeson, et al., 2011).*

Last but not least, IL-6 is considered to be involved in the development of hepatocellular carcinoma during obesity and colitis-associated cancer, two diseases that are related to inflammation (Grivennikov, et al., 2009; Gruber, et al., 2013; Pal, et al., 2014).

## 1.6. Genetic scores of obesity and aging/telomere length

Genetic Risk Score of obesity (GRS) is a characteristic number that results from 18 loci with 32 SNPs (single nucleotide polymorphisms) of the genes that are implicated to play important role in obesity (Speliotes, et al., 2010; Joseph, et al., 2018). That is accomplished using GWAS-Genome-Wide Association Study. GWAS, due to the huge amount of information, can provide very useful elements for research as its translational research could be used in clinical practice (Hindorff, et al., 2009). GRSs show the variation that exist in the genome and is related to this obesity risk. This results from the large number of SNPs, where each one allele is not so statistically significant for the whole outcome (Belsky, et al., 2013). Combination of these SNPs provide the total information based on genes with a normal distibution of values (Fisher, 1918). So, this score indicates genetic predisposition to obesity (Speliotes, et al., 2010). It is used in correlation with BMI because BMI is a marker for prediction of risk of a variety of diseases (Speliotes, et al. 2010).

As far as telomere length is concerned, mean telomere length in blood leukocytes (LTL) is smaller in men than women resulting in the development of many possible diseases, such as cardiovascular diseases (Codd, et al., 2013). Variants of telomere length at two loci on chromosomes have shown in GWAS a replicated association with mean LTL (Codd, et al., 2013). Genetic Risk Score for telomere length can be found by combining variants of different loci in the genes that are associated with cardiovascular diseases (Codd, et al., 2013). This has shown a relation of the alleles with shorter LTL with increased risk of cardiovascular diseases (Codd, et al., 2013). So, this score implicates the cardiological age of each person.

## 1.7. Objective

Purpose of master's thesis is the study of mechanisms of inflammation in obesity and aging. More specifically, after the correlation of circulating levels of white blood cells and lymphocyte subclasses between obese and non people, inflammatory cytokines that can be used as markers of obesity were found. Findings about possible methods (diet or exercise for example) for prevention of creating or maintaining inflammation could be helpful against human diseases. Also, the correlation of genetic scores both for obesity and aging with other characteristics like inflammation and cardiovascular diseases is a good idea for a better physical condition and human body weight regulation.

# 2. Methods

## 2.1. Study design

Two different groups of participants were used in this study. In the first group, 39 obese volunteers (BMI >30 kg/m$^2$) were included, while in the second group there were 27 lean (BMI<25 kg/m$^2$) healthy participants. In each volunteer leukocyte subpopulations were assessed.

Informed consent was obtained from all volunteers and venous blood was drawn into 10mL EDTA tubes. Leukocyte subpopulations count was determined either by a cell counter or by means of flow cytometry **(Figure 11)**.

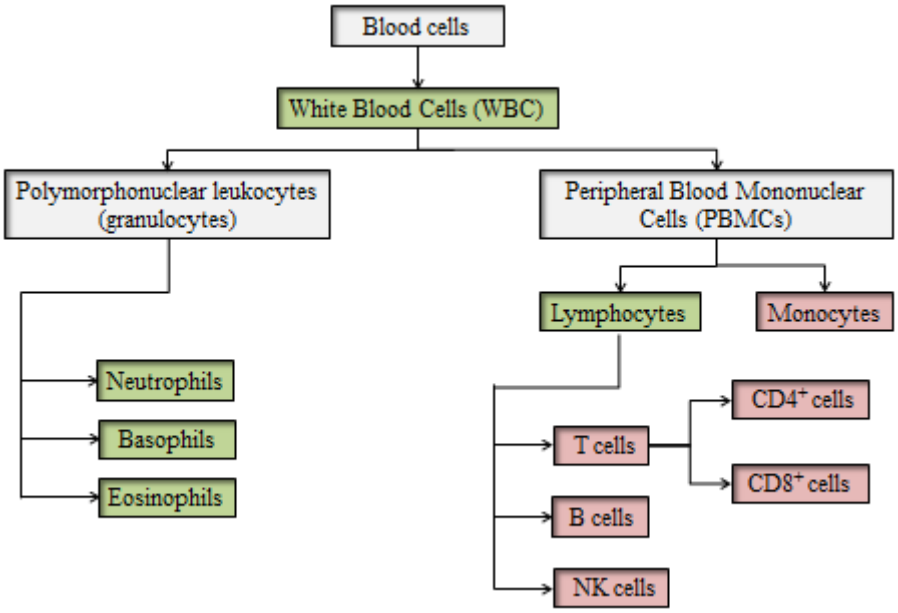


***Figure 11*** *Immune cells that are used in this study either by cell counter (green boxes) or by means of flow cytometry (red boxes). PBMC subpopulations were further analyzed by flow cytometry.*

## 2.2. Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral Blood Mononuclear Cells (PBMCs) consist of monocytes and lymphocytes **(Figure 11)**. The most commonly used method for the isolation of PBMCs is by density gradient centrifugation. This method is commonly used as immune cells can have different densities **(Figure 12)** and can be separated in distinct layers during centrifugation in the presence of a density gradient media, such as Ficoll-Paque medium. In this construction, cells that possess high density can migrate through

ficoll, while cells with a lower density will be found on top of the ficoll layer. As a result, PBMCs-containing layer can be easily extracted and purified.

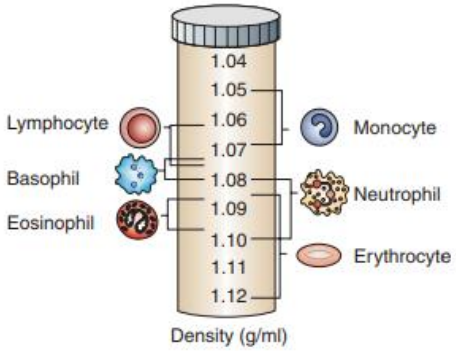


***Figure 12*** *Characteristic densities of each category of human blood cells (Munoz, & Leff, 2007).*

Peripheral blood was drawn in the presence of the anti-coagulant ethylenediamine-tetraacetic acid (EDTA) and then transferred to 50 ml falcon tube. It was diluted 1:1 with Roswell Park Memorial Institute 1640 (RPMI) and ficoll was overlaid with diluted blood slowly against the wall of tube (10 ml Ficoll for <30 ml diluted blood). Cells were separated by spinning at 900g for 20 min at 18$^{o}$C (no brake) and following that a Pasteur's pipette was used to collect the lymphocytes layer into a fresh tube **(Figure 13)**. Lymphocytes layer was topped up to 50 ml with RPMI, spinned at 300g for 10 min at 4$^{o}$C with brakes and were washed 2 more times. Cells were counted prior last wash using Neubauer's chamber by trypan blue exclusion. Then, they were resuspended in appropriate volume of pyrogen- free saline, namely PBS$^{+}$ (1% Human serum, 2mM EDTA) in order to be used for flow cytometry profiling.

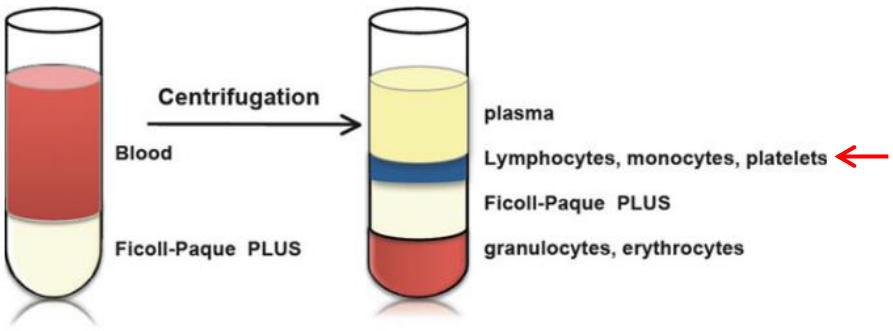


***Figure 13*** *PBMCs isolation. Peripheral blood is layered over ficoll and after centrifugation, blood components are separated into granulocytes and erythrocytes, ficoll, lymphocytes, monocytes (i.e. PBMCs) and platelets, and (4) plasma (Bharadwaj, et al., 2012).*

## 2.3. Immune profiling using flow cytometry

Flow cytometry is a widely used laser-based technique that checks each cell for the presence or absence of antigens (Craig, & Foon, 2013). This multiparameter method is used for the analysis of the expression of cell surface and intracellular molecules, the definition of cell size and volume, the detection of cell viability, and the characterization of different cell types in a mixed cell population. This method of observing and measuring features in each cell, is based on the measurement of fluorescence intensity emitted by fluorescent-labeled antibodies, meaning that cells after incubation in fluorochrome-bearing monoclonal antibodies will be imported in a solution, where they can move and pass from a place that laser light strikes and fluorescence is emitted (Kyriazis, 1995). The light is converted to electronics, is stored on computer and further analyzed (Kyriazis, 1995).

So, the three main components of a cytometer are:

- the fluidics system, that is responsible for sample transportation from the container to the flow cell where laser is placed,
- the optical system, that includes excitation light sources, lenses and filters responsible for light collection, as well as the detection system that gives rise to photocurrent generation,
- the electronics, which includes detectors and in which the photocurrent is digitized and prepared for further analysis

The sample is drawn from the tube and is injected into the flow chamber. There, laminar stream of sheath fluid focuses the cells through a nozzle and allows only the single pass of cells through the laser light at a time. Cells or particles scatter the light following their route through the laser beam. Forward scatter (FS) is measured by a detector placed in front of the light beam and is indicative of the size of the cell or particle passing in front of the laser. Side scatter (SS) is measured by several detectors placed to the side of the light beam and detects changes in optical density and complexity of the objects. Fluorescence produced by stained cells is detected by fluorescence detectors (Athanasopoulou, 2012) **(Figure 14)**.

Fluorescent dyes are used for measurement of fluorescence. Some of them can be FITC (fluorescein isothiocyanate), PE (phycoerythrin), ECD, PC5.5, PC7 (peridinin

chlorophyll protein) that absorb at 480nm, APC (allophycocyanin), APC-A700, APC-A750, that absorb at 633nm, Pacific Blue and Krome Orange, which absorb at 405nm (Kalina et al., 2012). Except for fluorescent dyes used in the experiment, fluorescence may also originate from autofluorescence within a cell. In order to determine the real signal emitted by an antibody, estimation of the background fluorescence is conducted by using unstained cells or isotype antibodies. Isotypes are antibodies with a binding domain that does not target any cell molecule and a constant region that may bind to some receptors on cell surface and dead cells. The isotype control should ideally be the same isotype, in terms of species, heavy and light chain, conjugated to the same fluorophore and have the same F:P ratio (i.e. a ratio indicative of the number of fluorescent molecules detected on an antibody) as the experimental antibody.

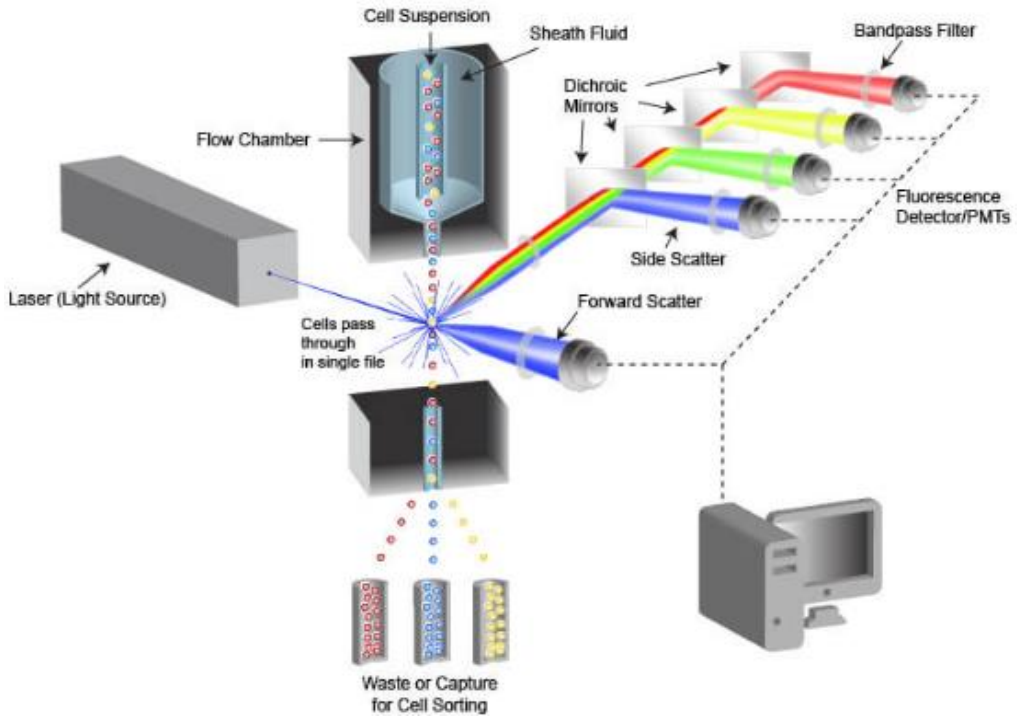


***Figure 14*** *Flow cytometry system [2].*

In the experiment, PBMCs were isolated for flow cytometry profiling according to that $2x10^6$ cell suspension in $PBS^+$ were dispensed in three different tubes. Antibody mastermix was prepared and 6μL of antibody mixture were added in the appropriate tube **(Table 1)**. The dilution of each monoclonal antibody in each tube was 1:10. Singles and isotype controls were also prepared. After 30min of incubation on ice in the dark, tubes were centrifuged at 1500rpm for 7 min at $4^oC$. Then, supernatants

were discarded and cells were resuspended in 150 μL $PBS^+$. DAPI (dilution 1:1000) was added when needed in order to exclude dead cells. Cells were then analyzed by flow cytometry (FACS Aria) and data acquisition and analysis were performed with the FACS Diva Software version 6.1.3.

***Table 1*** *Antibody mixture used for each staining (St1, St2, St3) for the detection of monocytes, NK cells and T cell subsets.*

| **St1**<br>**T cells** | **St2**<br>**$CD8^+$ T cells/**<br>**Tregs** | **St3**<br>**Monocytes/**<br>**NK cells** |
|---|---|---|
| CD3 FITC | CD3 FITC | CD45 PE/Dazzle |
| CXCR3 PE | CD4 PE | CD 14 PE |
| CD45RO APC | | CD16 FITC |
| CD4 PercPCy5.5 | CD127 PECy5 | CD15 PECy7 |
| CD45RA PECy7 | CD25 PECy7 | HLA-DR APCCy7 |
| CCR7 APCCy7 | CD8 APCCy7 | CD56 APC |

## 2.4. IL-6 ELISA

Blood was taken from each participant and was stored in tubes. 1ml blood from each sample was centrifuged and the supernatant that was isolated and stored in eppendorfs (aliquots) was serum. Also, 400 μl blood from each sample was used for the 48h treatment of cells. In other words, in 48-well plate 100 μl blood/cells from each participant was laid in each well, where in the two of the four wells that were used for each sample, 400 μl B culture medium were added, while in the other two remaining wells 400 μl BL culture medium were added. BL medium is characterized by the presence of 100 ng/ml LPS (Lipopolysaccharide) in order to induce cells in the conditions of obesity, while B medium is RPMI (10mM L-Glutamine, 10mM Pyruvate and 1X Penicillin-Streptomycin). After 48h incubation in 5% $CO_2$ and 37 $^oC$, the content of each well was collected in separate eppendorfs, centrifugation was done and supernatants were stored at -80 $^oC$.

IL-6 ELISA (Enzyme-Linked ImmunoSorbent Assay) was used for the analysis both of serum samples and cells after the induction of B and BL media. ELISA is the method that is used for the detection of a ligand (IL-6) in the samples by using antibodies that are raised against this ligand. So, for this purpose IL-6 ELISA from R & D systems was used in case of serum samples, whereas IL-6 ELISA High Sensitivity from Origene was used for the samples with the induction of B & BL media. In both kits the basic principle is that an antibody that is specific for IL-6 in humans is pre-coated in the whole 96-well plate. When samples, standards and possibly controls are added and are incubated for 2 or 3 hours, they bound on IL-6. Then, after washes a secondary or enzyme-linked polyclonal antibody specific for human IL-6 is added in which they bind to, and then any excess unbound reagent is removed after washes. If it is needed, an extra conjugation is done (depends on kit) and chromogen substrate is added resulting in the development of colour (blue) in each well proportionally to the amount of IL-6 that is bound to each sample. The colour development is stopped by the addition of acid as a result the colour is converted to yellow one and the intensity of the colour of each well is measured. The absorbance values that arise are used for the finding of concentration of IL-6 in each sample based on the standard curve that is generated from standard samples, which were created following manufacturer's protocol. Standard curve results from the calculating difference of absorbance values from the primary to the reference wavelength, the calculation of the average value for each standard as they were in duplicates and the correspondence of each absorbance with the known concentration of each sample. Based on the equation of standard curve, the concentrations of the samples (pg/ml) were measured. It is worth noting that samples were not duplicates.

## 2.5. Genetic Risk Scores in obesity

Peripheral whole blood was collected from participants and DNA was extracted using the Applied Biosystems OpenArrayTaqman genotyping platform. Samples were genotyped in duplicate and assigned using ABI's Genotyper software for OpenArrayTaqman data. 18 loci with 32 SNPs for BMI based on the study of Speliotes, et al., 2010 and 16 rare single nucleotide variants (SNVs) associated with BMI using exome-targeted genotyping arrays based on Turcot, et al., 2018 were selected. Genetic Risk Score was created using the calculation of an average score per

SNP and SNV. This score is calculated by multiplying the weight of each SNP/SNV (from the studies of Speliotes, et al., 2010 and Turcot, et al., 2018 respectively) with the number of risk alleles for that SNP/SNV. Finally, GRS is standarized using the mean and standard deviation of each sample. It is worth noting that gene-based meta-analyses were held to assemble rare and low-frequency coding SNVs (Turcot, et al., 2018). So, five rare SNVs in three genes (KSR2, 2 in MC4R, 2 in GIPR) and eleven low-frequency SNVs in ten genes (ZBTB7B, 2 in ACHE, RAPGEF3, PRKAG1, RAB21, HIP1R, ZFHX3, ENTPD6, ZFR2 and ZNF169) were used for the analysis (Turcot, et al., 2018).

## 2.6. Genetic Risk Score for telomere length

Peripheral blood from each participant was collected and mean telomere length of each sample was measured through quantitative PCR-based technique (Cawthon, 2009). Based on this method telomere length is expressed as a ratio of telomere repeat length (T) to copy number of a single gene (S). Furthermore, DNA samples were genotyped for seven SNPs (rs11125529, rs10936599, rs7675998, rs2736100, rs9420907, rs8105767 and rs755017) using TaqMan Pre-Designed SNP genotyping assays. To check the association of these variants with the risk of cardiovascular disease and TL, a multiple SNP risk score analysis was held. More specifically, based on Codd, et al., 2013, SNP on chromosome 2p16.2 (rs11125529-ACYP2) was very close to the threshold for genome-wide significance, while SNP in the locus on 16q23.3 (rs2967374-MPHOSPH6) has almost lost this threshold (Codd, et al., 2013). The most significantly associated locus was the TERC locus (rs10936599) (Codd, et al., 2013). Also, four further loci rs2736100-TERT, rs7675998-NAF1, rs9420907-OBFC1 and rs755017-RTEL1 are associated loci (Codd, et al., 2013). Statistical analysis was performed using Spearman's q correlation coefficients. The threshold for statistically significance was set at p-value$< 0.05$.

## 2.7. Statistical analysis

GraphPad Prism 8 was used for graph construction and statistical analysis of results. All the analyses (such as white blood cell percentages) are expressed as mean±standard error of the mean (SEM). Statistical analysis was performed using the

unpaired Mann-Whitney t-test (two- tailed) when comparing cell absolute counts and percentages derived from obese and lean participants. Accordingly, Mann-Whitney t-test was used in the analyses of pro-inflammatory cytokine and BMI, age, sex, smoking, physical exercise and coffee and alcohol consumption, correlation test for XY graphs (two-tailed) for genetic risk scores of obesity and telomere length and their correlations, while multiple t-test per row and nested graphs were used for the analysis of IL-6 conecntartions in the samples that are induced by B or BL medium. One-way Anova test was used for the correlation of IL-6 and genetic risk score for telomere length (2 quantitative parameters). Lastly, data and graphs for pro-inflammatory cytokine and its parameters BMI, coffee consumption and physical activity were analysed by IBM SPSS Statistics using Sperman's correlation. The threshold for statistically significance was set at $p\text{-value} < 0.05$.

# 3. Results

## 3.1. Participants' characteristics

In the study, participants were classified based on their BMI (Body Mass Index). So, there were included 27 lean (BMI=22.06±0.36 kg/$m^2$) and 39 obese participants (BMI= 35.54±1.04 kg/$m^2$). 18 from 27 lean participants were females (66,67%) and 9 were males (33,33%), while 28 from 39 obese (71,8%) samples were females and 11 were males (28,2%). From the 66 total number of participants 46 (69,69%) of them were females and 20 (30,30%) were males **(Table 2)**.

***Table 3*** *Baseline characteristics of the participants included in the study (*: mean±standard error of the mean (SEM), SEM=Standard Deviation/Square Root of the Count of Cells that contain numbers).*

| | **Lean** | **Obese** |
|---|---|---|
| **No.** | 27 | 39 |
| **Sex ratio, F/M** | 18/9 | 28/11 |
| **Age, years*** | 35.5±2.6037 | 43.3077±2.03019 |
| **Weight, kg*** | 61.36±1.881 | 97.81±3.331 |
| **BMI, kg/$m^2$*** | 21.94±0.3432 | 35.34±98.75 |

The majority of lean participants was at age 20-39 years with a mean age at 35.5±2.6037 years, while 30-49 years were the privilege age for obese participants, whose mean age was 43.3077±2.03019 years meaning that obese patients are older than lean ones or the sample that was selected was not so uniform **(Figures 15, 16 and 17A)**.

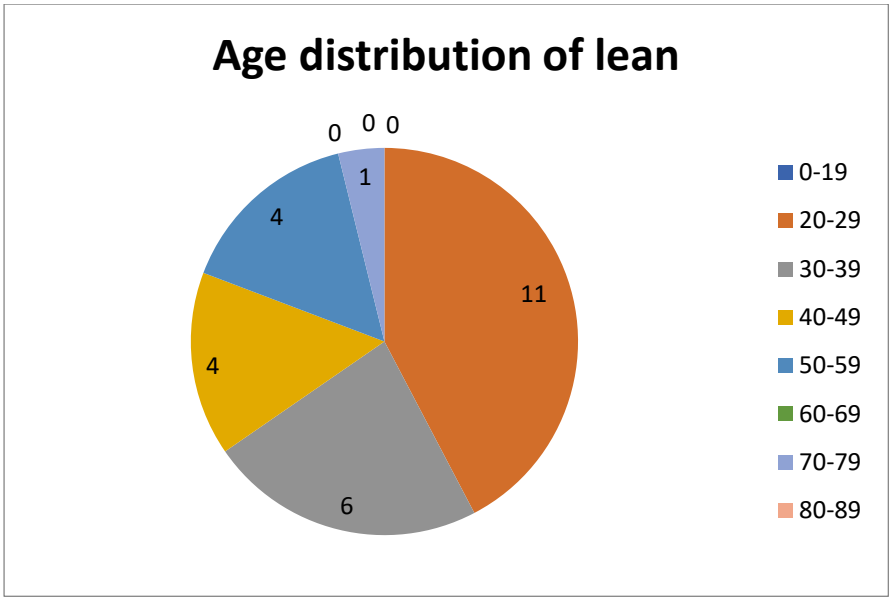


***Figure 15*** *Age distribution of lean participants (years).*

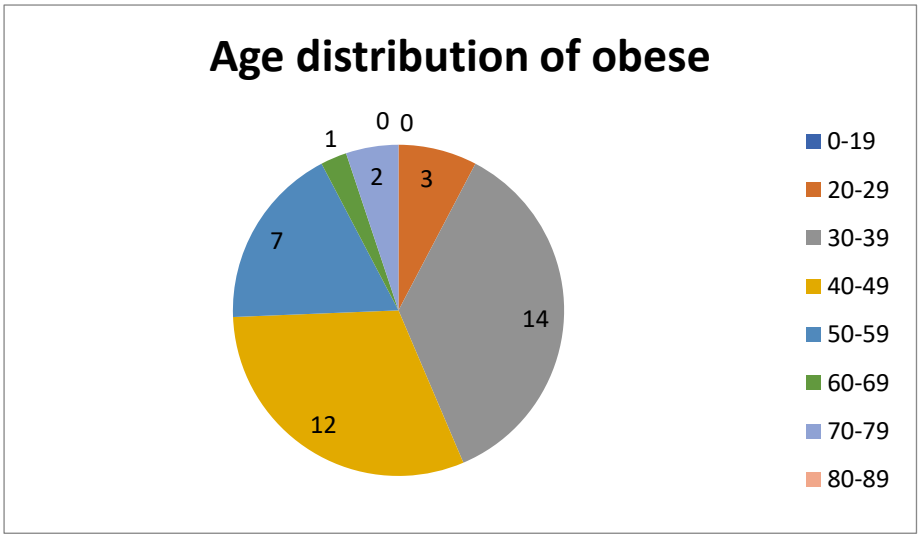


***Figure 16*** *Age distribution of obese participants (years).*

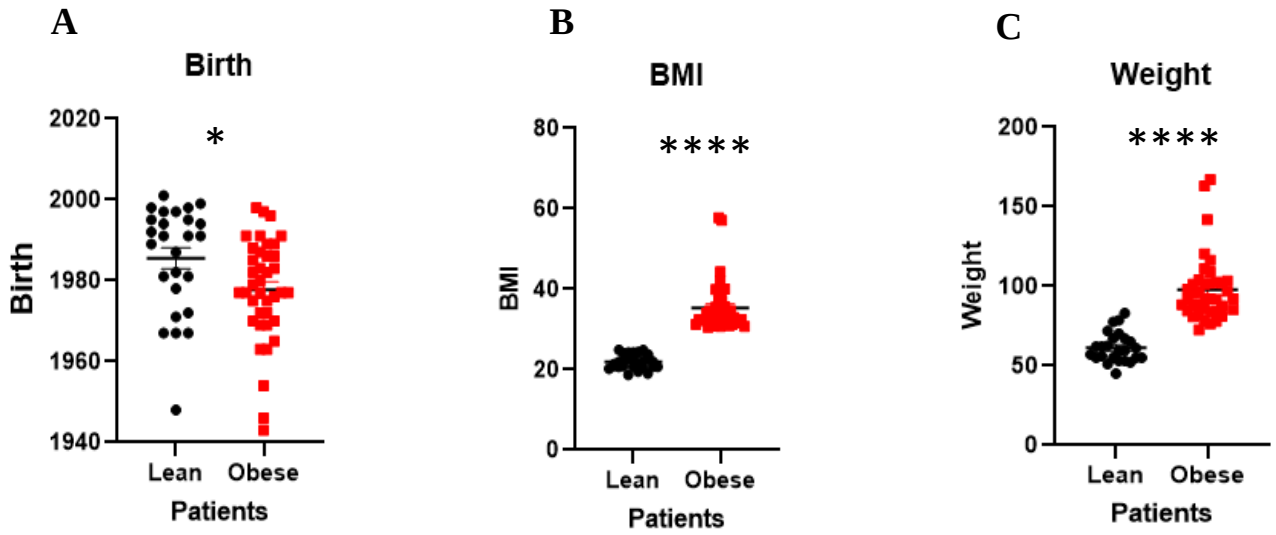


***Figure 17*** *Correlation of BMI-two subcategories: lean and obese with A. birth of participants (*p-value=0.0080<0.05), B. BMI and C. weight of participants (****p-value=<0.0001).*

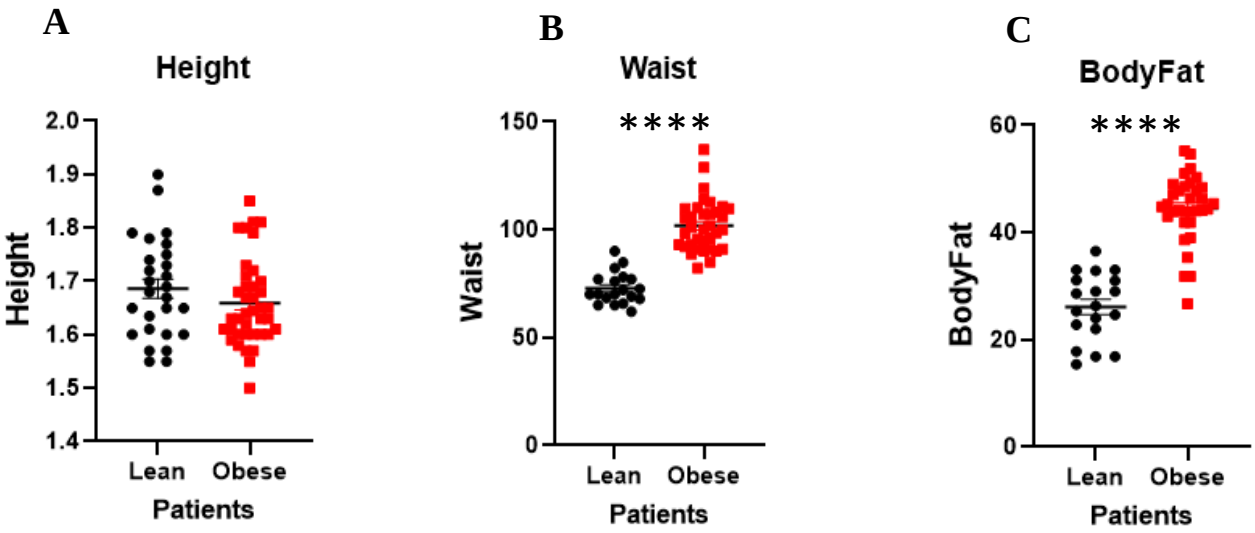


***Figure 18*** *Correlation of BMI-two subcategories: lean and obese with A. height of participants (*p-value=0.2731>0.05), B. waist and C. bodyfat of participants (****p-value=<0.0001).*

It is observed that the levels of weight, waist and bodyfat of participants are notably increased in obese subjects compared to lean ones (with statistically significant values), while height of obese participants has smaller values than lean ones, justifying the elevated numbers of BMI in obese people **(Figures 17B, C, 18)**.

## 3.2. Gating strategy in flow cytometry profiling

In order to evaluate the number of immune cells present during obesity, three gating strategies were followed.

**Staining 1**

In this strategy, the following cell populations were assessed **(Figure 19)**:

- T cells ($CD3^+$)
- $CD4^+$ T cells
- Naïve T cells ($CD45RA^+CCR7^+$)
- Central memory T cells ($T_{CM}$) ($CD45RO^+CCR7^+$)
- Effector memory T cells ($T_{EM}$) ($CD45RO^+CCR7^-$)
- Terminally differentiated effector memory cells (TEMRA) ($CD45RA^+CCR7^-$)
- $CD45RA^+CD45RO^+$ T cells
- $CD45RA^+CXCR3^+$ T cells
- $CD45RO^+CXCR3^+$ T cells

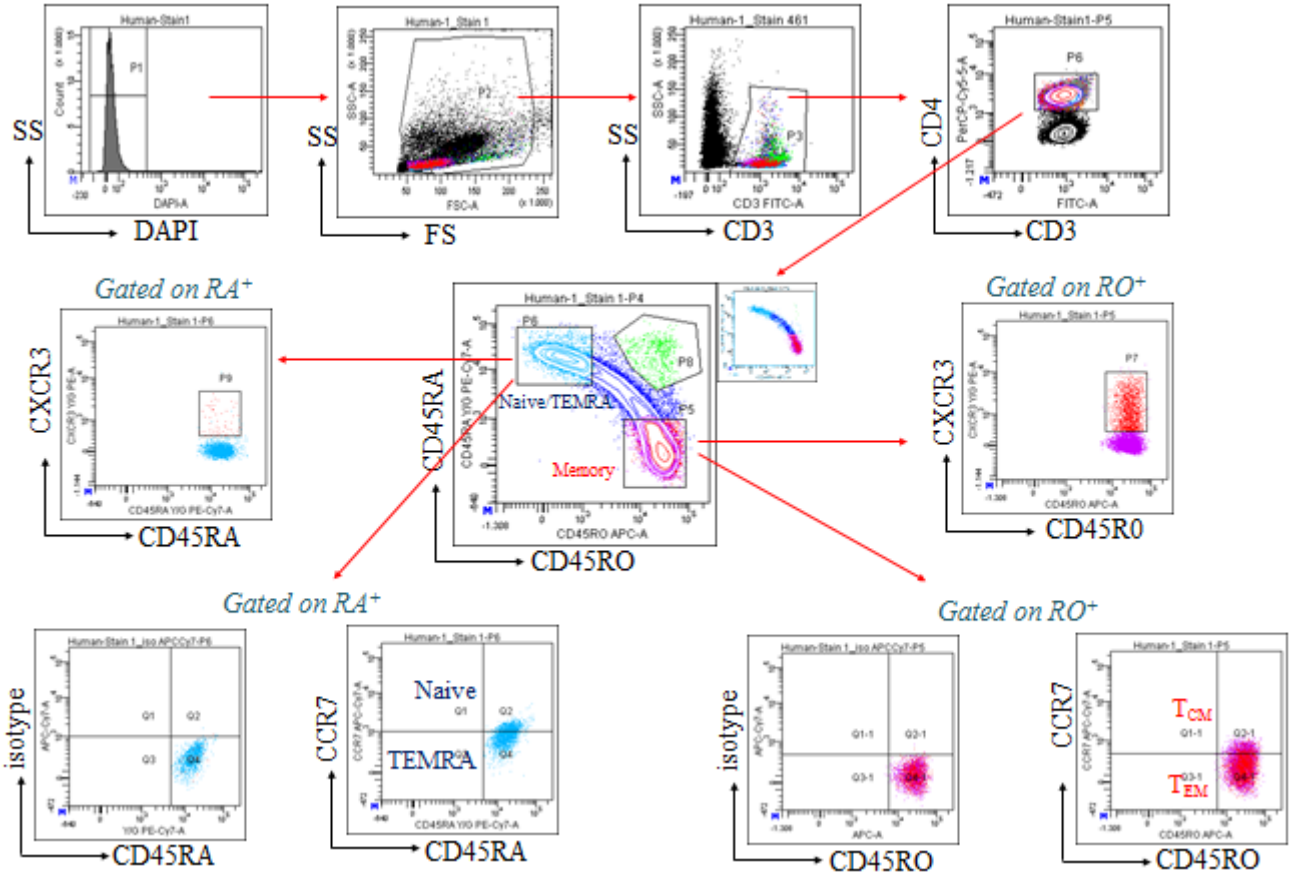


***Figure 19*** *Gating strategy followed for the assessment of $CD4^+$ T cells subtypes (Staining 1).*

**Staining 2**

In this strategy, the following cell populations were assessed **(Figure 20)**:

- T cells ($CD3^+$)
- $CD4^+$ T cells
- T cytotoxic cells ($CD8^+$)
- Regulatory T cells (Tregs) ($CD127^-CD25^+$)

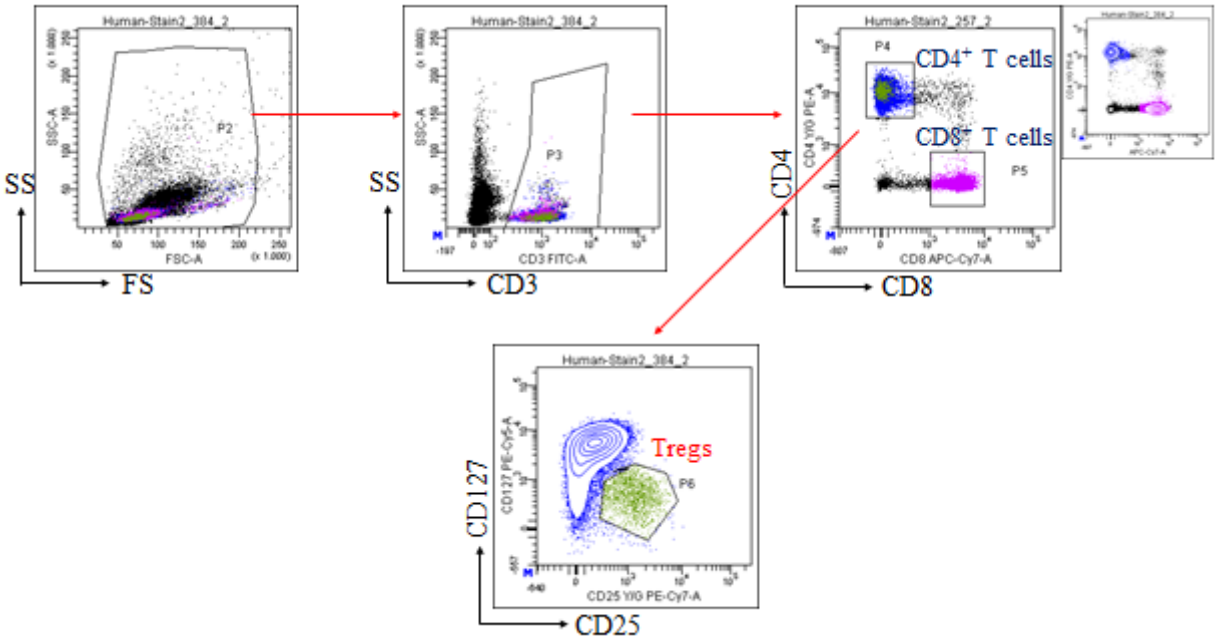


***Figure 20*** *Gating strategy followed for the assessment of $CD8^+$ T cells and T regulatory cells (Tregs) (Staining 2).*

**Staining 3**

In this strategy, the following cell populations were evaluated **(Figure 21)**:

- Monocytes

- Classical ($CD14^+CD16^-$)
- Intermediate ($CD14^+CD16^+$)
- $CD14^{low}CD16^+$
- Non- classical ($CD14^-CD16^+$)

- NK cells

- $CD56^{+}CD16^{+}$
- $CD56^{bright}CD16^{-}$
- $CD56^{dim}CD16^{-}$

- B lymphocytes ($CD16^{-}CD14^{-}HLA\text{-}DR^{+}$)

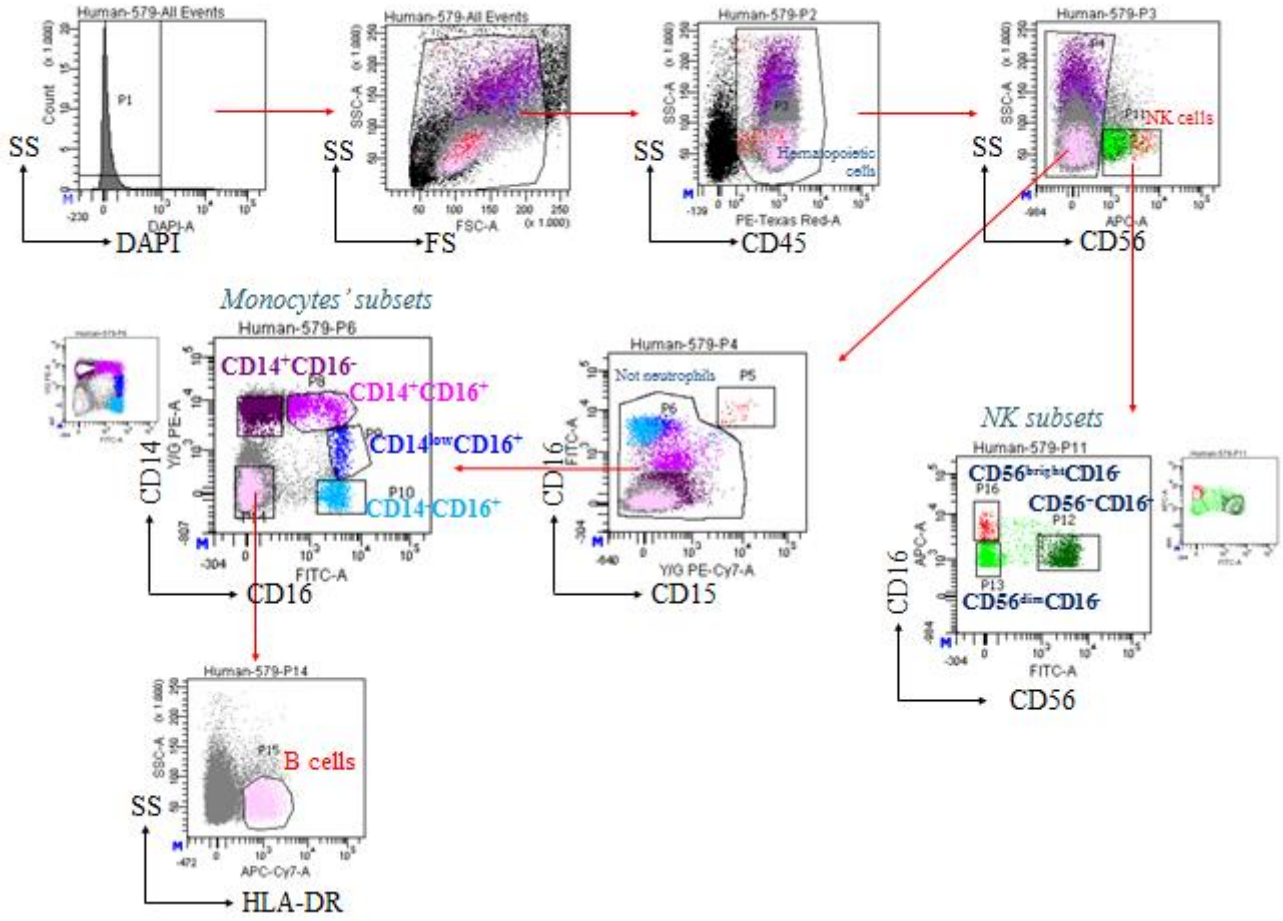


***Figure 21*** *Gating strategy followed for the assessment of B lymphocytes, monocytes and NK subsets (Staining 3).*

## 3.3. Differences in immune cell numbers between lean and obese individuals

Differences in absolute numbers and percentages of immune cells between obese and lean participants are summarized in **Table 3**.

***Table 4*** *Leukocyte and lymphocyte subset counts in lean and obese individuals. Values are presented as mean±SEM. (*: p values in bold represent statistically significant differences between obese and control group, p<0.05 is the threshold).*

| **Type of cell** | **Lean** | **Obese** | |
|---|---|---|---|
| | **mean±SEM** | | **p value*** |
| **White blood cells (cells/µL)** | 6070±266.9 | 6802±347.4 | 0.1784 |
| **Basophils (% total cells)** | 0.7658±0.09475 | 0.6475±0.05774 | 0.2372 |
| **Basophils (x $10^3$/µl)** | 0.09176±0.03780 | 0.04542±0.006993 | 0.7970 |
| **Eosinophils (% total cells)** | 2.676±0.4520 | 2.303±0.2059 | 0.9240 |
| **Eosinophils (x $10^3$/µl)** | 0.2282±0.05912 | 0.1812±0.02470 | 0.8972 |
| **Neutrophils (% total cells)** | 55.87±1.856 | 55.17±1.465 | 0.8600 |
| **Neutrophils (x $10^3$/µl)** | 3.127±0.2401 | 4.060±0.3419 | **0.0453** |
| **PBMCs (total cells)** | 2105x$10^4$±1642506 | 27188235±1526403 | **0.0091** |
| **$CD15^+CD16^+CD56^-$ Neutrophils (% total cells)** | 2.312±0.5329 | 2.339±0.5146 | 0.8399 |
| **$CD15^+CD16^+CD45^+$ Neutrophils (% total cells)** | 2.017±0.4554 | 2.061±0.4001 | 0.9849 |
| **Not Neutrophils $CD56^-$ (% total cells)** | 97.14±0.6075 | 97.11±0.5740 | 0.7999 |
| **Not Neutrophils $CD456^+$ (% total cells)** | 87.91±1.632 | 90.02±1.315 | 0.2998 |
| **$CD14^+CD16^-$Not Neutrophils (Monocytes) (% total cells)** | 14.15±1.215 | 11.87±0.7228 | 0.2252 |
| **$CD14^+CD16^-$Not NK (Monocytes) (% total cells)** | 13.79±1.160 | 11.58±0.8202 | 0.2129 |

| | | | |
|---|---|---|---|
| **$CD14^{+}CD16^{-}CD45^{+}$ (Monocytes) (% total cells)** | 12.23±1.035 | 10.36±0.5727 | 0.2790 |
| **$CD14^{+}CD16^{+}$Not Neutrophils (Monocytes) (% total cells)** | 1.681±0.2553 | 1.909±0.2400 | 0.4826 |
| **$CD14^{+}CD16^{+}$Not NK (Monocytes) (% total cells)** | 1.623±0.2448 | 1.817±0.2764 | 0.6959 |
| **$CD14^{+}CD16^{+}CD45^{+}$ (Monocytes) (% total cells)** | 1.419±0.2105 | 1.688±0.2163 | 0.3765 |
| **$CD14^{low}CD16^{+}$Not Neutrophils (Monocytes) (% total cells)** | 0.8762±0.1123 | 1.179±0.1406 | 0.1031 |
| **$CD14^{low}CD16^{+}$Not NK (Monocytes) (% total cells)** | 0.8452±0.1060 | 1.110±0.1445 | 0.1385 |
| **$CD14^{low}CD16^{+}CD45^{+}$ (Monocytes) (% total cells)** | 0.7295±0.08779 | 1.046±12.49 | 0.0566 |
| **$CD14^{-}CD16^{+}$Not Neutrophils (Monocytes) (% total cells)** | 3.942±0.7479 | 4.300±0.6145 | 0.6035 |
| **$CD14^{-}CD16^{+}$Not NK (Monocytes) (% total cells)** | 3.877±0.7463 | 3.220±0.4529 | 0.7802 |
| **$CD14^{-}CD16^{+}CD45^{+}$ (Monocytes) (% total cells)** | 3.402±0.6644 | 3.910±0.5955 | 0.5774 |
| **$CD14^{-}CD16^{-}$Not Neutrophils (% total cells)** | 72.02±1.889 | 73.99±1.429 | 0.7019 |
| **$CD14^{-}CD16^{-}$Not NK (% total cells)** | 70.38±1.975 | 73.41±1.576 | 0.5678 |
| **$CD14^{-}CD16^{-}CD45^{+}$ (% total cells)** | 62.44±2.001 | 65.44±1.651 | 0.3807 |
| **Lymphocytes (% total cells)** | 33.95±1.578 | 34.85±1.341 | 0.5607 |
| **Lymphocytes (x 103/µl)** | 1.929±0.1060 | 2.380±0.1806 | **0.0286** |
| **T ($CD3^{+}$) lymphocytes (absolute number of total PBMCs)** | 325836±65673 | 580925±68268 | **0.0085** |
| **$CD14^{-}CD16^{-}HLADR^{+}$ B lymphocytes (% $CD14^{-}CD16^{-}$)** | 11.22±0.9147 | 12.18±0.7874 | 0.4441 |

| | | | |
|---|---|---|---|
| **$CD14^{-}CD16^{-}HLADR^{+}$Not Neutrophils B lymphocytes (%)** | 8.031±0.6545 | 8.832±0.5129 | 0.3102 |
| **$CD14^{-}CD16^{-}HLADR^{+}$Not NK B lymphocytes (%)** | 12.36±1.867 | 15.00±3.187 | 0.8083 |
| **$CD14^{-}CD16^{-}HLADR^{+}$ B lymphocytes (% $CD45^{+}$)** | 11.15±1.808 | 12.36±2.339 | 0.9598 |
| **$CD56^{+}CD45^{+}$ NK cells (% total PBMCs)** | 8.535±0.8148 | 9.352±1.096 | 0.6431 |
| **$CD56^{bright}CD16^{-}CD56^{+}$NK cells (% $CD56^{+}$)** | 5.200±0.8254 | 4.056±0.6549 | 0.6430 |
| **$CD56^{bright}CD16^{-}CD45^{+}$ NK cells (% $CD45^{+}$)** | 0.3510±0.04912 | 0.3644±0.06676 | 0.6304 |
| **$CD56^{dim}CD16^{-}$ $CD56^{+}$ NK cells (% $CD56^{+}$)** | 19.77±3.214 | 24.10±3.294 | 0.2132 |
| **$CD56^{dim}CD16^{-}CD45^{+}$ NK cells (% $CD45^{+}$)** | 1.390±0.1828 | 1.942±0.3486 | 0.1571 |
| **$CD56^{+}CD16^{+}CD56^{+}$ NK cells (%)** | 66.22±4.174 | 64.75±3.627 | 0.6494 |
| **$CD56^{+}CD16^{+}CD45^{+}$ NK cells (%)** | 6.124±0.7665 | 6.424±0.9628 | 0.8289 |
| **$CD3^{+}CD4^{+}$ (% total PBMCs)** | 60.33±1.593 | 64.03±1.418 | 0.0716 |
| **$CD3^{+}CD4^{+}$ (% $CD3^{+}$ cells)** | 59.91±1.619 | 65.01±1.409 | **0.0142** |
| **$CD3^{+}CD8^{+}$ (%)** | 28.48±1.785 | 24.05±1.029 | **0.0476** |
| **$CD3^{+}CD4^{+}CD45RA^{+}CD45RO^{-}$ (% $CD3^{+}$)** | 21.81±1.976 | 18.81±1.182 | 0.3522 |
| **$CD3^{+}CD4^{+}CD45RA^{+}CD45RO^{-}$ (% $CD4^{+}$)** | 35.55±2.497 | 29.48±1.783 | 0.0841 |
| **$CD3^{+}CD4^{+}CD45RA^{-}CD45RO^{+}$ (% $CD3^{+}$)** | 25.18±1.191 | 29.46±1.305 | **0.0391** |
| **$CD3^{+}CD4^{+}CD45RA^{-}CD45RO^{+}$ (% total $CD4^{+}$)** | 42.52±2.182 | 45.80±1.558 | **0.0124** |

| | | | |
|---|---|---|---|
| **$CD3^+CD4^+CD45RA^+CD45RO^+$ (% $CD3^+$)** | 0.8926±0.1185 | 1.149±0.1249 | 0.2063 |
| **$CD3^+CD4^+CD45RA^+CD45RO^+$ (% total $CD4^+$)** | 1.463±0.1868 | 1.824±0.1913 | 0.2552 |
| **$CD3^+CD4^+CD45RA^+CCR7^+$ (Naïve) (% $CD3^+$)** | 4.044±0.4856 | 4.699±0.5390 | 0.5190 |
| **$CD3^+CD4^+CD45RA^+CCR7^+$ (Naïve) (% $CD4^+$)** | 6.901±0.8805 | 7.356±0.8236 | 0.9447 |
| **$CD3^+CD4^+CD45RA^+CCR7^+$ (Naïve) (% $CD45RA^+$)** | 21.43±2.945 | 26.19±2.673 | 0.2556 |
| **$CD3^+CD4^+CD45RA^+CCR7^-$ (TEMRA) (% $CD3^+$)** | 18.16±1.930 | 21.82±1.441 | 0.1039 |
| **$CD3^+CD4^+CD45RA^+CCR7^-$ (TEMRA) (% $CD4^+$)** | 46.04±2.622 | 46.91±1.926 | 0.7561 |
| **$CD3^+CD4^+CD45RA^+CCR7^-$ (TEMRA) (% $CD45RA^+$)** | 78.37±2.963 | 73.36±2.648 | 0.2186 |
| **$CD3^+CD4^+CD45RO^+CCR7^+$ (CM) (% $CD3^+$)** | 0.6893±0.092628 | 1.294±0.1851 | 0.0644 |
| **$CD3^+CD4^+CD45RO^+CCR7^+$ (CM) (% $CD4^+$)** | 1.174±0.1662 | 2.028±0.2984 | 0.0794 |
| **$CD3^+CD4^+CD45RO^+CCR7^+$ (CM) (% $CD45RO^+$)** | 2.770±0.3320 | 4.342±0.6037 | 0.2086 |
| **$CD3^+CD4^+CD45RO^+CCR7^-$ (EM) (% $CD3^+$)** | 24.26±1.126 | 27.92±1.230 | 0.0800 |
| **$CD3^+CD4^+CD45RO^+CCR7^-$ (EM) (% $CD4^+$)** | 41.56±3.024 | 43.51±1.525 | 0.4526 |
| **$CD3^+CD4^+CD45RO^+CCR7^-$ (EM) (% $CD45RO^+$)** | 96.51±0.3657 | 94.83±0.6658 | 0.3155 |
| **$CD3^+CD4^+CD45RA^+CXCR3^+$ (% $CD3^+$)** | 0.8670±0.1557 | 0.6939±0.06280 | 0.6746 |
| **$CD3^+CD4^+CD45RA^+CXCR3^+$ (% $CD4^+$)** | 1.391±0.2116 | 1.068±0.08922 | 0.3487 |
| **$CD3^+CD4^+CD45RA^+CXCR3^+$ (% $CD45RA^+$)** | 3.774±0.3806 | 4.032±0.4952 | 0.9710 |

| | | | |
|---|---|---|---|
| **$CD3^+CD4^+CD45RO^+CXCR3^+$ (% $CD3^+$)** | 11.24±0.7440 | 11.00±0.9140 | 0.6177 |
| **$CD3^+CD4^+CD45RO^+CXCR3^+$ (% $CD4^+$)** | 18.76±1.213 | 16.96±1.232 | 0.3352 |
| **$CD3^+CD4^+CD45RO^+CXCR3^+$ (% $CD45RO^+$)** | 44.79±2.075 | 36.66±2.314 | **0.0193** |
| **$CD3^+CD4^+CD127^-CD25^+$ (T regs) (% $CD3^+$)** | 4.380±0.1467 | 4.253±0.2467 | 0.3861 |
| **$CD3^+CD4^+CD127^-CD25^+$ (T regs) (% $CD4^+$)** | 7.496±0.4002 | 6.597±0.3497 | 0.0715 |

**White blood cells (WBC)**

White blood cells in participants' blood were found to be increased in obese (mean±SEM=6802±347.4 cells/μl) compared to lean participants (mean±SEM=6070±266.9 cells/μl) despite the fact that this result is not statistically significant **(Figure 22)**.

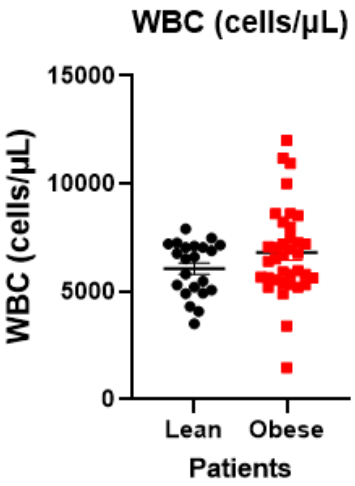


***Figure 22*** *Differences of white blood cell counts between lean and obese participants (p-value=0.1784>0.05).*

### 3.3.1. Polymorphonuclear leukocytes (granulocytes)

As far as polymorphonuclear leukocytes are concerned, there is a reduction in basophil **(Figure 23C, D)** and eosinophil levels **(Figure 23E, F)** in obese compared to lean participants, while a slight increase in neutrophils' levels is observed in obese ones **(Figure 23A, B)**. Only the result of absolute numbers in neutrophil counts is statistically significant.

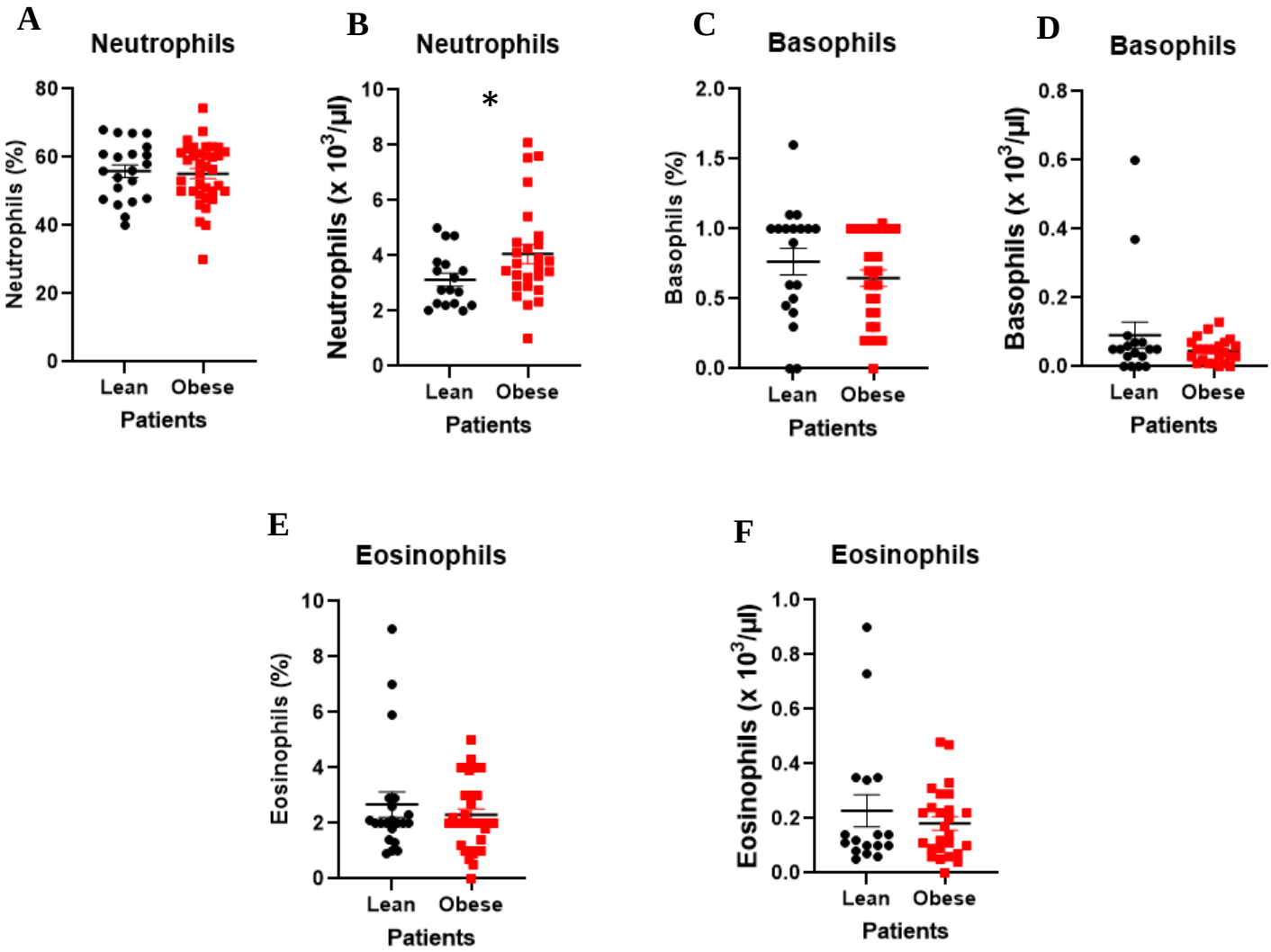


***Figure 23*** *Differences of percentages and absolute numbers in A./B. neutrophils counts (p-values=0.86/0.0453), C./D. basophils counts (p-values=0.2372/0.7970) and E./F. eosinophils (p-values=0.9240/0.8972) between lean and obese participants. The threshold of statistical significance is p-value<0.05 (*).*

*3.3.1.1. Neutrophils*

Neutrophils are characterized by CD15, CD16 markers. Their percentages for their relation with $CD56^-$ and $CD45^+$ are the following:

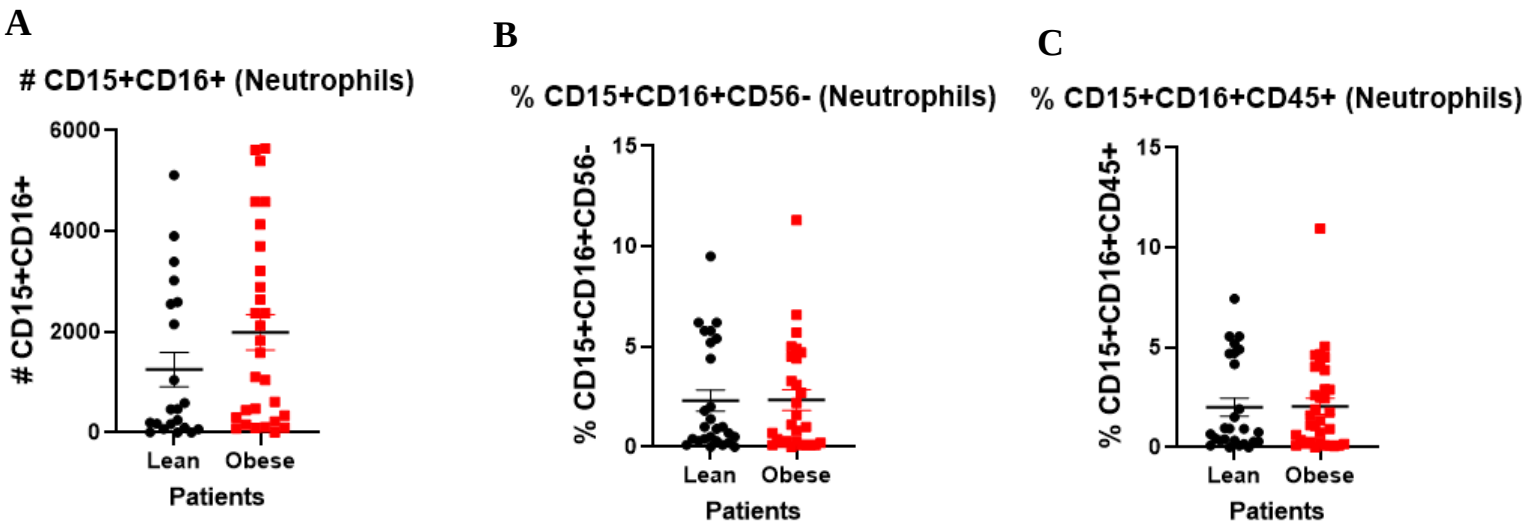


***Figure 24*** *Differences of A. absolute number of neutrophils (p-value=0.1107), percentages of neutrophils gated to B. $CD56^-$ (p-value=0.8399) and C. $CD45^+$ (p-value=0.9849).*

It is observed that only in the case of neutrophil counts, there is an increase in their levels in obese participants compared to non-obese ones **(Figure 24A)**. In percentages of neutrophils that are gated either for $CD56^-$ marker or for $CD45^+$, no difference between lean and obese patients is observed **(Figure 24B, C)**.

3.3.2. Peripheral Blood Mononuclear Cells (PBMCs)

Circulating numbers of PBMCs were significantly higher in obese compared to lean individuals **(Figure 25)**.

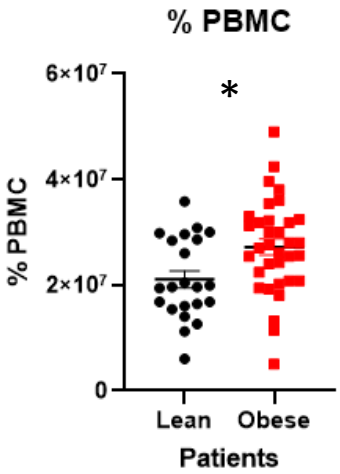


***Figure 25*** *Differences of PBMCs in obese and lean participants (*p-value=0.0091<0.05).*

*3.3.2.1. Monocytes*

Monocytes are divided in four main subcategories: classical monocytes-$CD14^{+}CD16^{-}$, non-classical monocytes-$CD14^{-}CD16^{+}$, intermediate monocytes-$CD14^{+}CD16^{+}$ and $CD14^{low}CD16^{+}$ monocytes.

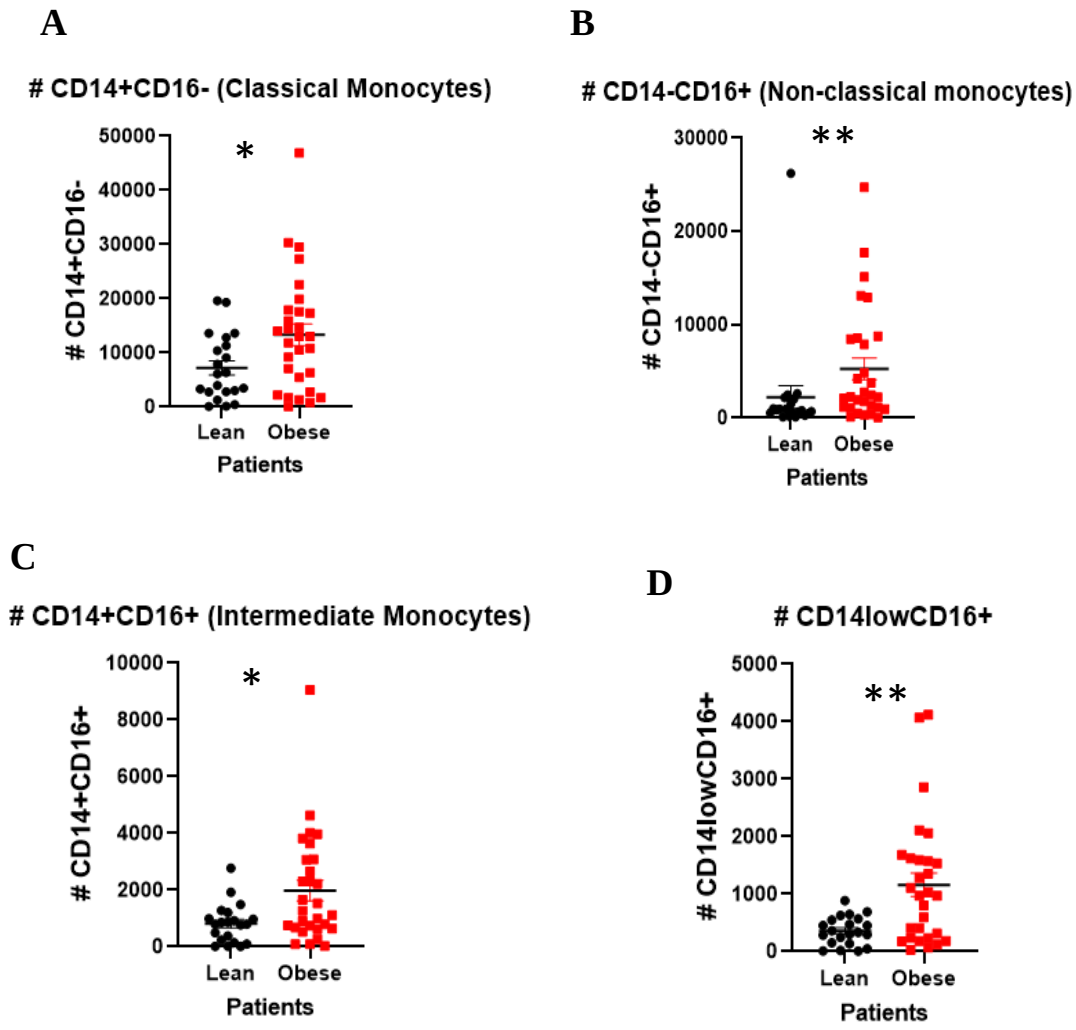


***Figure 26*** *Differences in absolute numbers of three subcategories of monocytes between lean and obese subjects A. Classical monocytes ($CD14^{+}CD16^{-}$) (*p-value=0.0377<0.05), B. Non-classical monocytes ($CD14^{-}CD16^{+}$) (**p-value=0.0049<0.05), C. Intermediate monocytes ($CD14^{+}CD16^{+}$) (*p-value=0.0219<0.05) and D.$CD14^{low}CD16^{+}$ monocytes (**p-value=0.0042<0.05).*

As for monocytes' absolute numbers, a statistically significant increase in each type of monocytes in obese people compared to non-obese ones is observed. More specifically, $CD14^{+}CD16^{-}$ (classical) (mean±SEM=7207±1312 vs. 13315±1995) **(Figure 26A)**, $CD14^{-}CD16^{+}$ (non-classical) (mean±SEM=2243±1210 vs. 5260±1147) **(Figure 26B)** and $CD14^{+}CD16^{+}$ (intermediate) (mean±SEM=809.8±150 vs. 1980±357.6) **(Figure 26C)** and $CD14^{low}CD16^{+}$ monocytes (mean±SEM=358.5±54

vs. 1162±202) **(Figure 26D)** subsets were found elevated in obese compared to lean individuals.

The percentages of these different categories of monocytes are gated for 3 markers, $CD45^{+}$, Not NK ($CD56^{-}$) and Not Neutrophils ($CD15\text{-}CD16^{-}$).

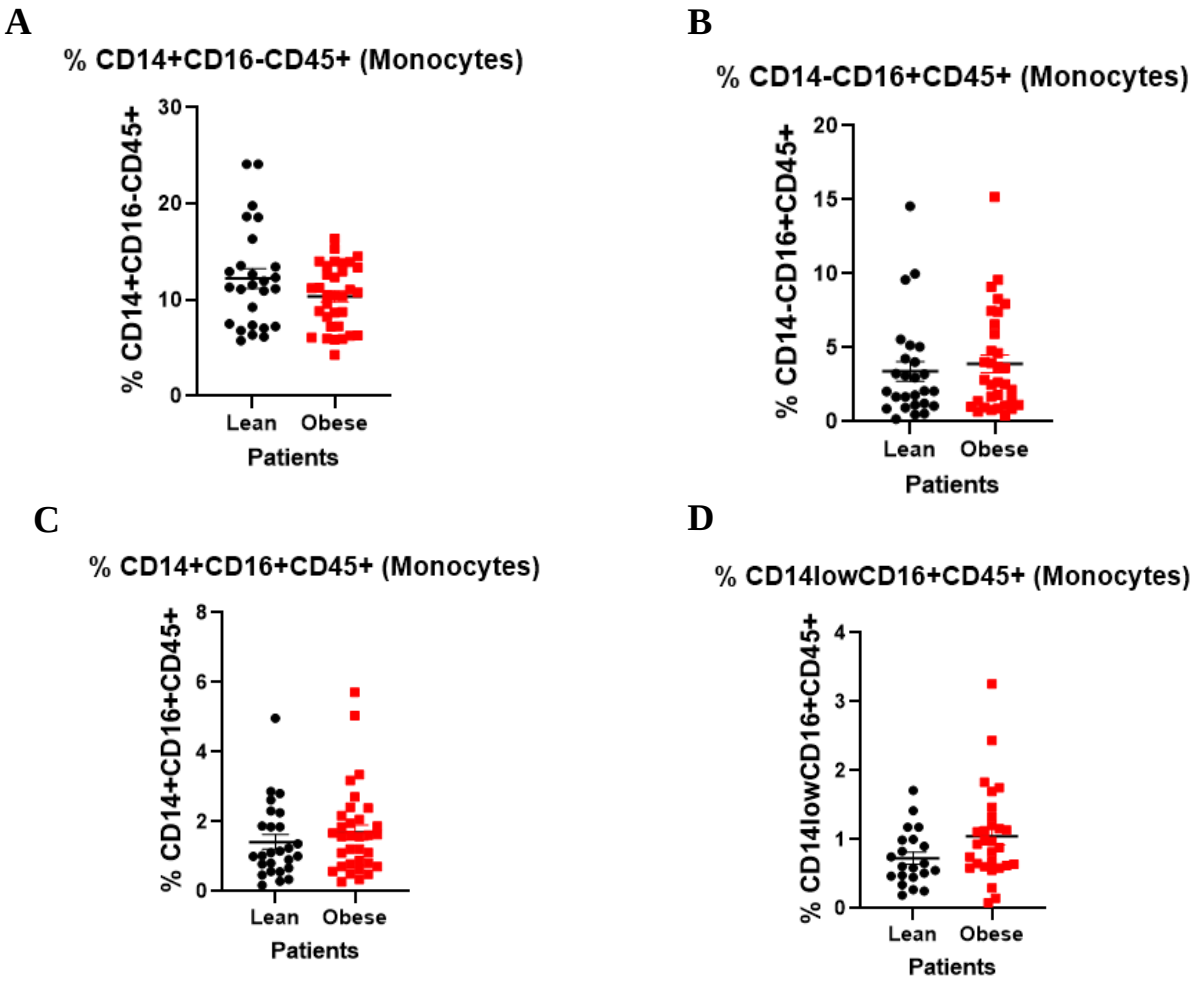


***Figure 27*** *Differences in percentages of counts of A. Classical monocytes (p-value=0.2790), B. Non-classical monocytes (p-value=0.5774), C. Intermediate monocytes (p-value=0.3765) and D. $CD14^{low}CD16^{+}$ (p-value=0.0566) gated on $CD45^{+}$ marker between lean and obese participants.*

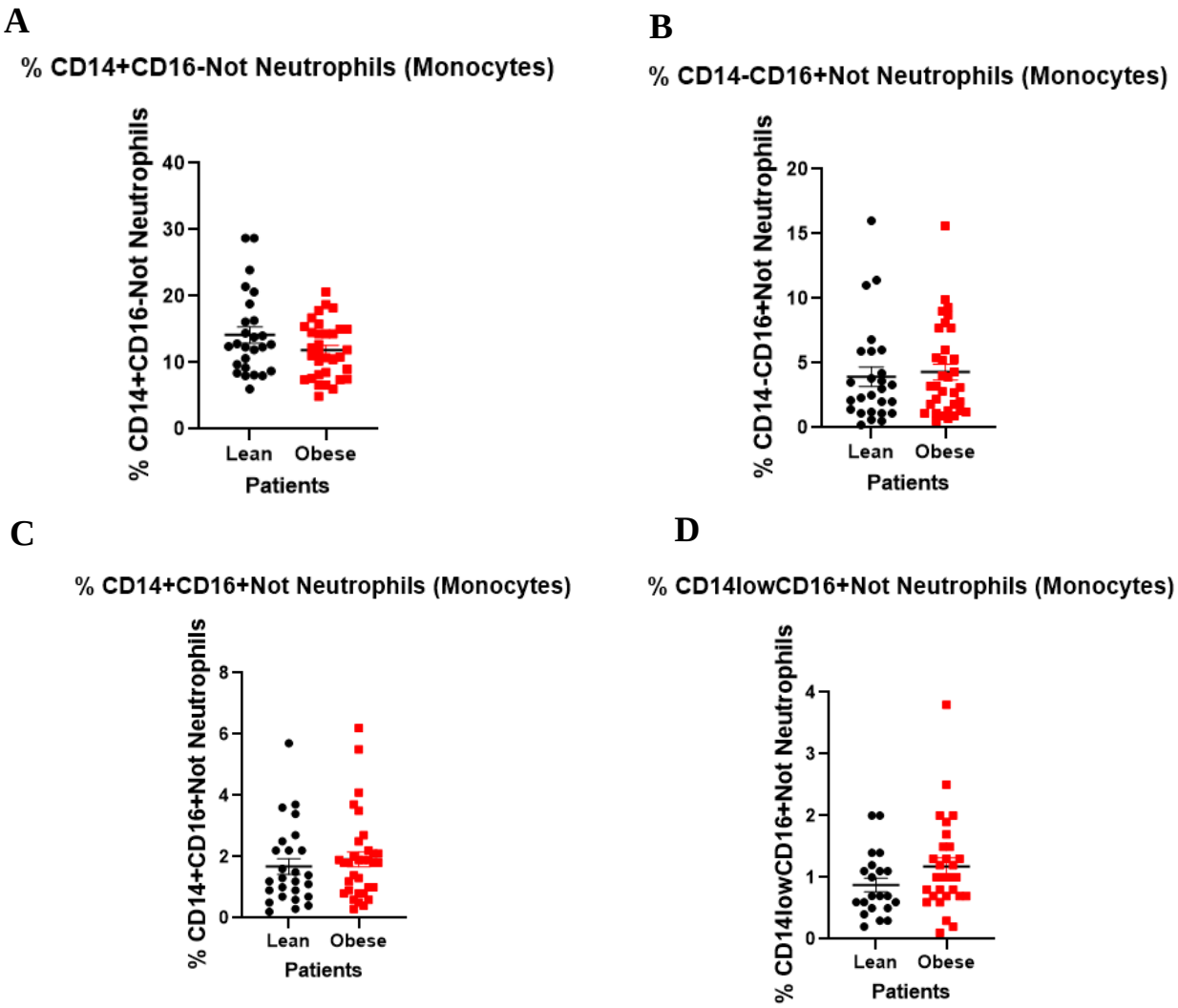


***Figure 28*** *Differences in percentages of counts of A. Classical monocytes (p-value=0.2252), B. Non-classical monocytes (p-value=0.5774), C. Intermediate monocytes (p-value=0.6035) and D.* $CD14^{low}CD16^{+}$ *(p-value=0.1431) gated on* $CD15^{-}$$CD16^{-}$ *marker (Not Neutrophil) between lean and obese participants.*

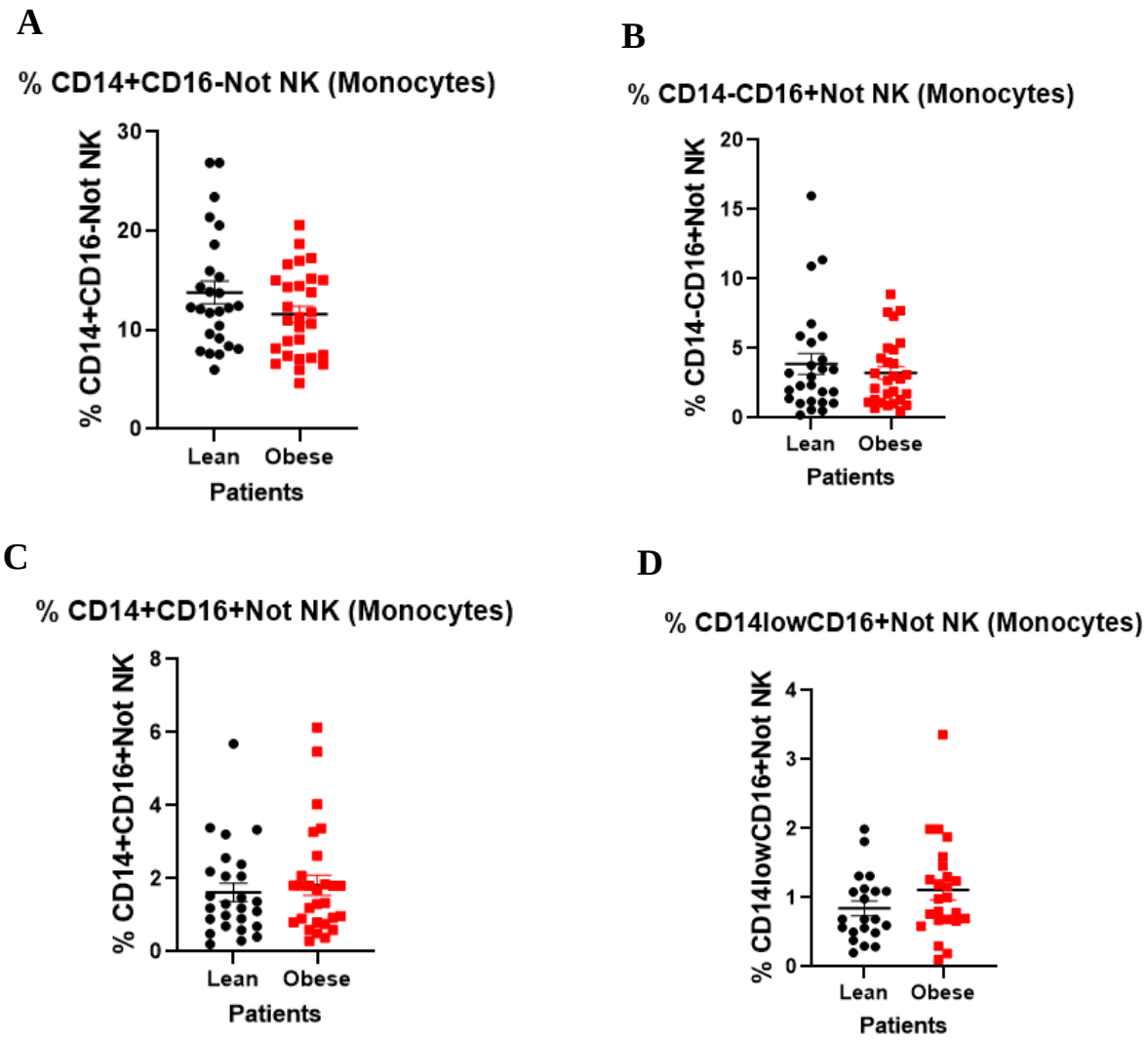


***Figure 29*** *Differences in percentages of counts of A. Classical monocytes (p-value=0.2129), B. Non-classical monocytes (p-value=0.78024), C. Intermediate monocytes (p-value=0.6959) and D. CD14$^{low}$CD16$^{+}$ (p-value=0.1385) gated on CD56$^{-}$ marker (Not NK) between lean and obese participants.*

Classical monocytes gated for all three markers present a reduction in their levels in obese participants compared to lean ones **(Figures 27, 28, 29 A)**. In the levels of non-classical and intermediate monocytes there is a slightly increase in obese subjects **(Figures 27B,C 28B,C, 29C)** except from non-classical monocytes gated for the marker Not NK (CD56$^{-}$), where a slightly reduction in obese people is observed **(Figure 29B)**. Last, but not least, CD14$^{low}$CD16$^{+}$ monocytes gated for all markers present the most significant increases (without having though statistical significance) in their levels that are found in obese people compared to non-obese ones **(Figures 27, 28, 29 D)**.

*3.3.2.2. Lymphocytes*

Lymphocytes' total percentage and absolute numbers (mean±SEM=1.929±0.1060 vs. 2.380±0.1806 x $10^3$) are slightly increased in obese compared to lean participants **(Figure 30A, B)**. The subcategories of T, B and NK cells are analysed in the following figures.

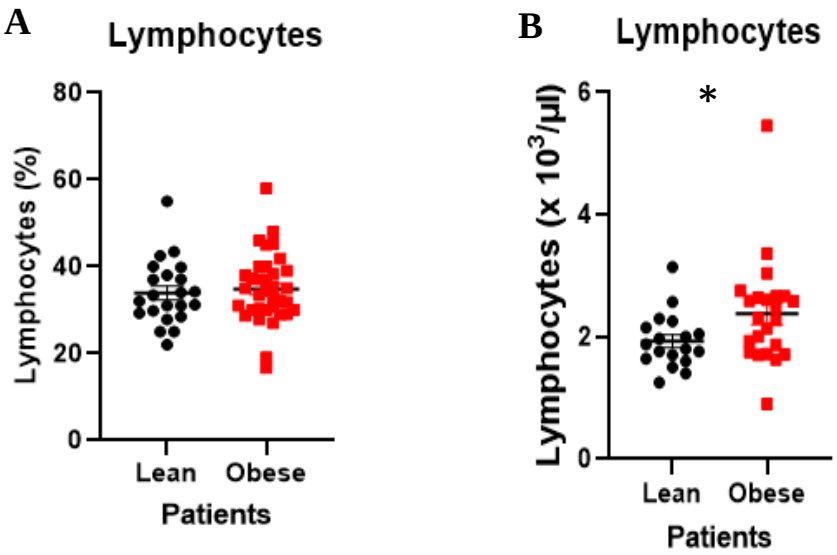


***Figure 30*** *Differences of percentages and absolute numbers lymphocyte counts in obese and lean participants (p-value=0.5607, *p-value=0.0286<0.05).*

3.3.2.2.1. T lymphocyte compartment

In obese individuals, it is observed that total levels of $CD4^+$ (mean±SEM=198559±42229 vs. 390900±44306), $CD3^+$ (mean±SEM=325836±65673 vs. 580925±68268), $CD8^+$ (mean±SEM=91632±18901 vs. 144312±17890) T cells and T regulatory cells (Tregs) (mean±SEM=13330±2429 vs. 25284±3112) are much higher than those of controls **(Figure 31)**.

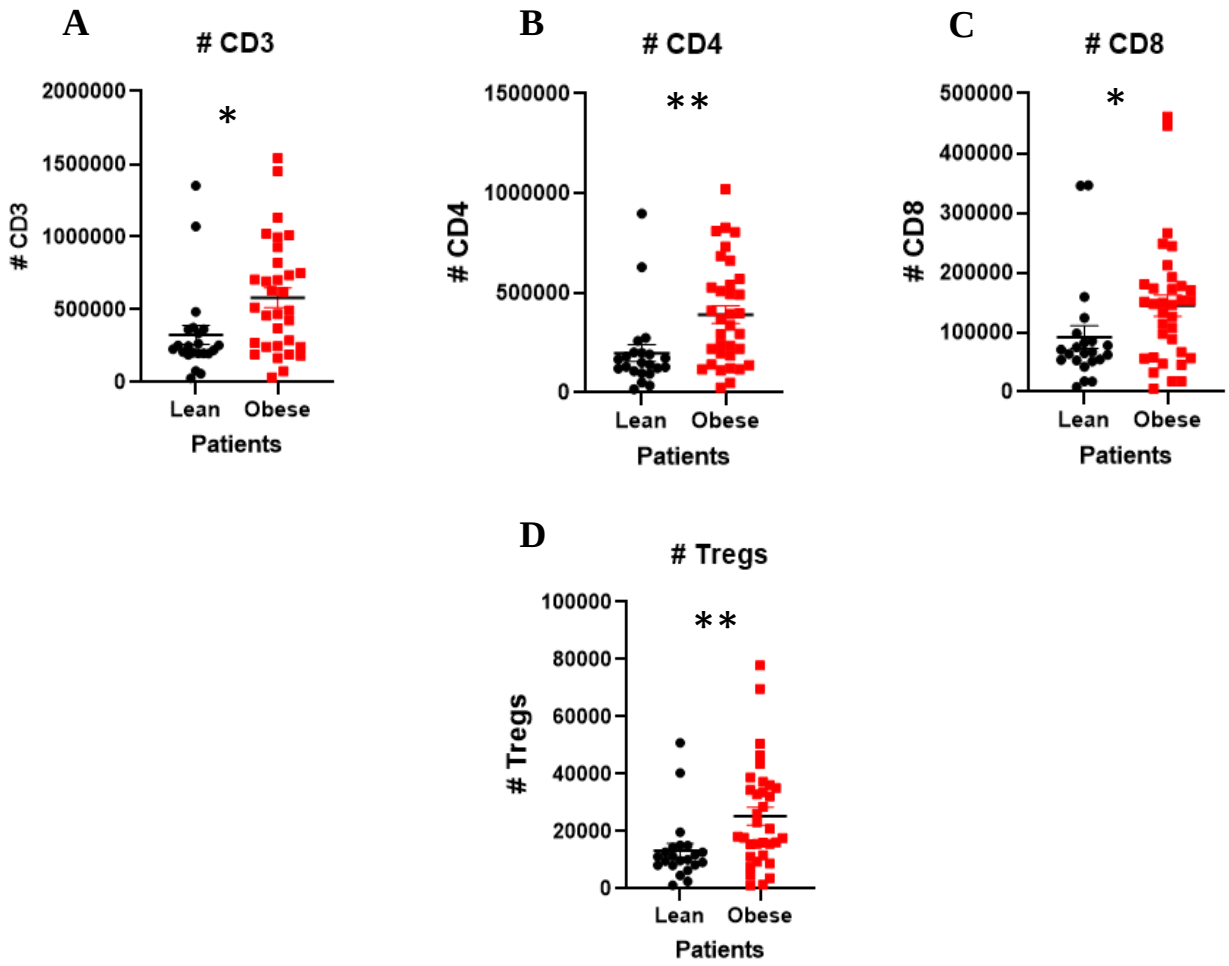


***Figure 31*** *Differences of absolute numbers in different categories of T cells between lean and obese participants A. $CD3^+$ T cells (**p-value=0.0085), B. $CD4^+$ T cells (**p-value=0.0015), C. $CD8^+$ T cells (*p-value=0.0161) and D. T regulatory cells (**p-value=0.0029). All values are statistically significant ($p<0.05$).*

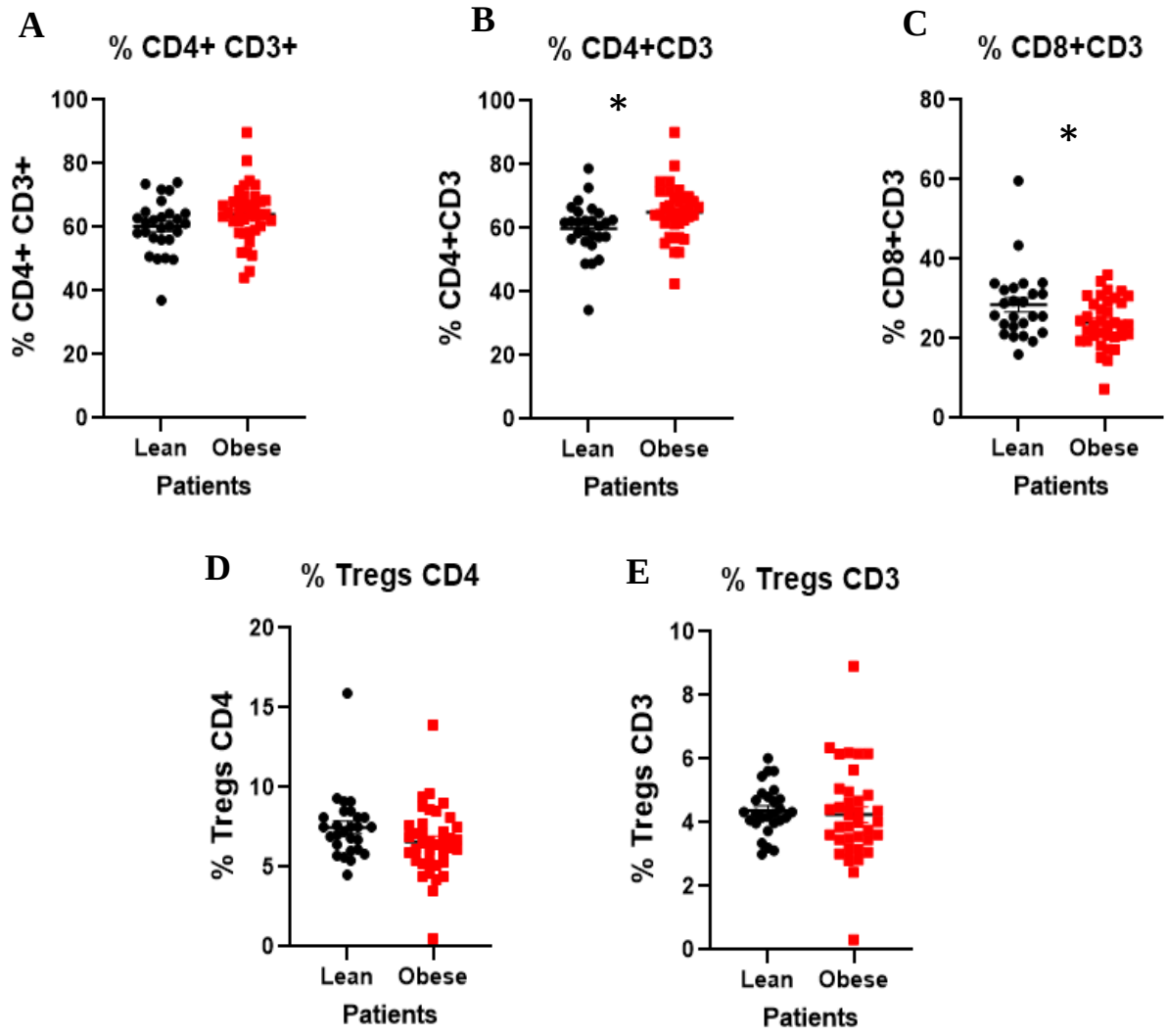


***Figure 32*** *Differences of percentages of counts in different categories of T cells between lean and obese participants A. $CD4^+CD3^+$ T cells (p-value=0.0716), B. $CD4^+CD3^+$ T cells gated for $CD3^+$ T cells (*p-value=0.0142<0.05), C. $CD3^+CD8^+$ T cells (*p-value=0.0476<0.05), D. $TregCD4^+$ (p-value=0.0715) and E. $TregCD3^+$ (p-value=0.3861).*

In cases of $CD4^+CD3^+$ T cells, the levels are elevated in obese participants compared to non-obese ones. As far as $CD8^+CD3^+$ T cells ($CD8^+$ T cells gated for $CD3^+$) are concerned, their levels are decreased in obese patients, while in $TregCD4^+$ (gated for $CD4^+$) there is a slightly reduction in obese ones and in $TregCD3^+$ (gated for $CD3^+$) no difference is observed **(Figure 32)**. So, the anti-inflammatory Treg cells, which

levels were elevated in obese compared to lean participants, are decreased in the obese group regarding their distribution in $CD4^+$ T cell compartment (Figures 31, 32 D).

**$CD4^+$ T cells compartment**

$CD4^+$ T cells are further assessed based on the expression of CD45RA/CD45RO markers and the expression of the marker CCR7 is used for the classification of cells belonging to CD45RA and CD45RO compartment. More specifically, absolute numbers of $CD45RA^+CCR7^+/CCR7^-$ T cells (Naïve/TEMRA (terminally differentiated effector memory)) (mean±SEM= 288663±44332 vs. 591156±93833/ mean±SEM=1236488±174176 vs. 2743971±274037) are statistically significant increased in obese individuals **(Figure 33A, B)**. The levels of $CD45RO^+$ $CCR7^+/CCR7^-$ T cells (Central/Effector Memory) (mean±SEM=46557±7532 vs. 142362±22079/ mean±SEM=2978250±309219 vs. 4672059±373120) are elevated in obese participants and the difference between the obese and control group reaches the

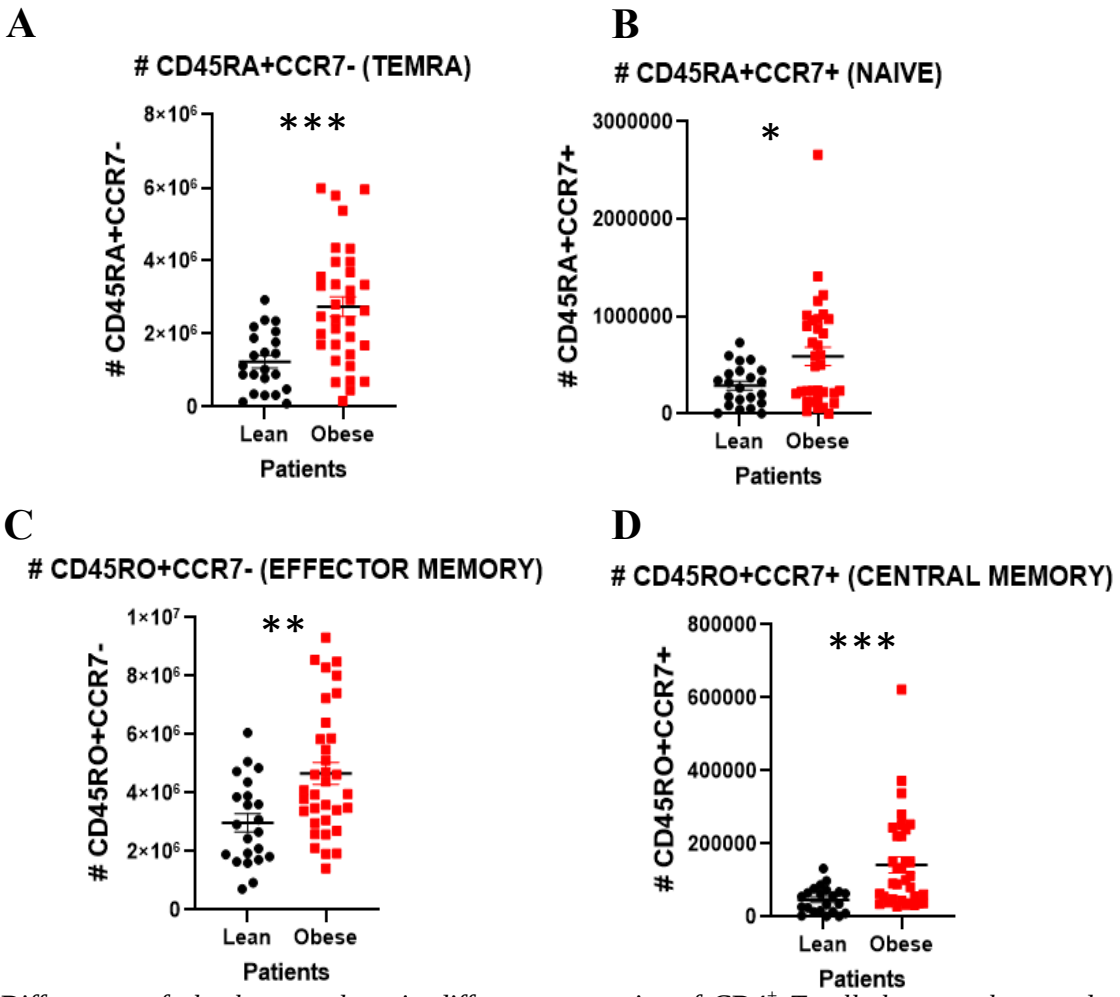


***Figure 33*** *Differences of absolute numbers in different categories of $CD4^+$ T cells between lean and obese participants A. $CD45RA^+CCR7^-$ (TEMRA) T cells (***p-value=0.0002), B. $CD45RA^+CCR7^+$ T cells (NAÏVE) (*p-value=0.0387), C. $CD45RO^+CCR7^-$ (Effector Memory) T cells (**p-value=0.0039) and D. $CD45RO^+CCR7^+$ (Central Memory) (***p-value=0.0003). All the values are statistically significant ($p<0.05$).*

levels of statistical significance **(Figure 33C, D)**.

Regarding their distribution in $CD4^+$ T cell compartment, naïve T cells are slightly increased in obese group **(Figure 34B)**, whereas TEMRA T cells are about the same at both groups **(Figure 34A)**. As far as central or effector memory T cells are concerned, both total cell percentages are elevated in obese people compared to non-obese ones **(Figure 34C, D)**.

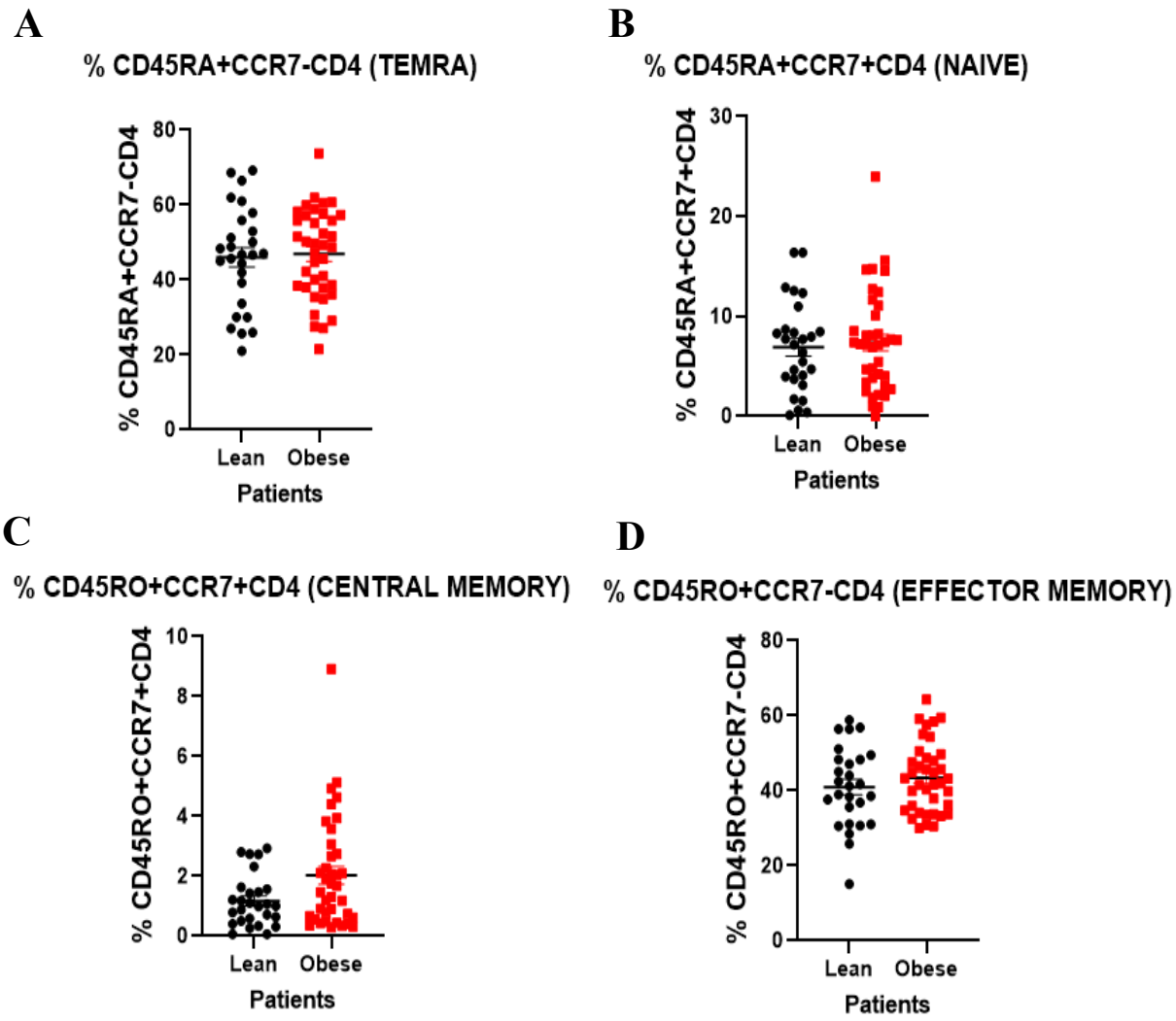


***Figure 34*** *Differences of percentages of numbers in different categories of $CD4^+$ T cells regarding their distribution to $CD4^+$ T cells between lean and obese participants A. $CD45RA^+CCR7^-CD4$ (TEMRA) T cells (p-value=0.7561), B. $CD45RA^+CCR7^+CD4$ T cells (NAÏVE) (p-value=0.9447), C. $CD45RO^+CCR7^+CD4$ (Central Memory) (p-value=0.4526) and D. $CD45RO^+CCR7^-CD4$ (Effector Memory) T cells (p-value=0.0794). All the values are not statistically significant ($p<0.05$).*

Regarding their distribution in $CD3^+$ T cell compartment, naïve T cells are increased in obese group **(Figure 35B)**, whereas TEMRA T cells are about the same at both groups **(Figure 35A)**. As far as central or effector memory T cells are concerned, both total cell percentages are elevated in obese people compared to non-obese ones **(Figure 35C, D)**.

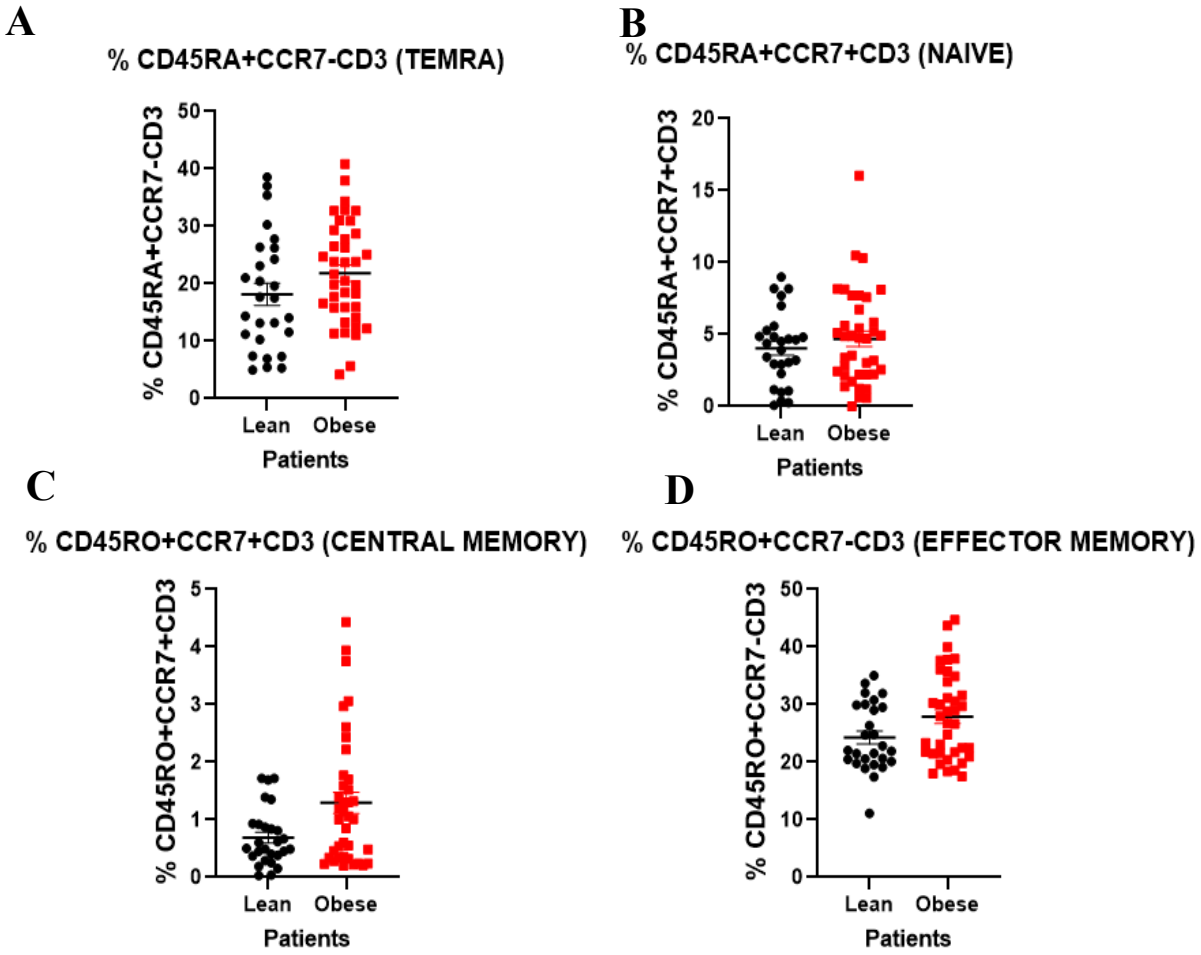


***Figure 35*** *Differences of percentages of numbers in different categories of $CD4^+$ T cells regarding their distribution to $CD3^+$ T cells between lean and obese participants A. $CD45RA^+CCR7^-CD3$ (TEMRA) T cells (p-value=0.1039), B. $CD45RA^+CCR7^+CD3$ T cells (NAÏVE) (p-value=0.5190), C. $CD45RO^+CCR7^+CD3$ (Central Memory) (p-value=0.0800) and D. $CD45RO^+CCR7^-CD3$ (Effector Memory) T cells (p-value=0.0644). All the values are not statistically significant ($p<0.05$).*

When TEMRA and naïve cell levels are examined regarding their distribution in $CD45RA^+$ T cell compartment, slightly increased levels of $CD45RA^+CCR7^+CD45RA^+$ cells are observed in obese patients **(Figure 36B)**, while $CD45RA^+CCR7^-CD45RA^+$ cells have no difference between the two groups **(Figure 36A)**. However, $CD45RO^+CCR7^+CD45RO^+$ cells are found to be increased in obese subjects **(Figure 36C)**, while levels of $CD45RO^+CCR7^-CD45RO^+$ cells are decreased **(Figure 36D)**.

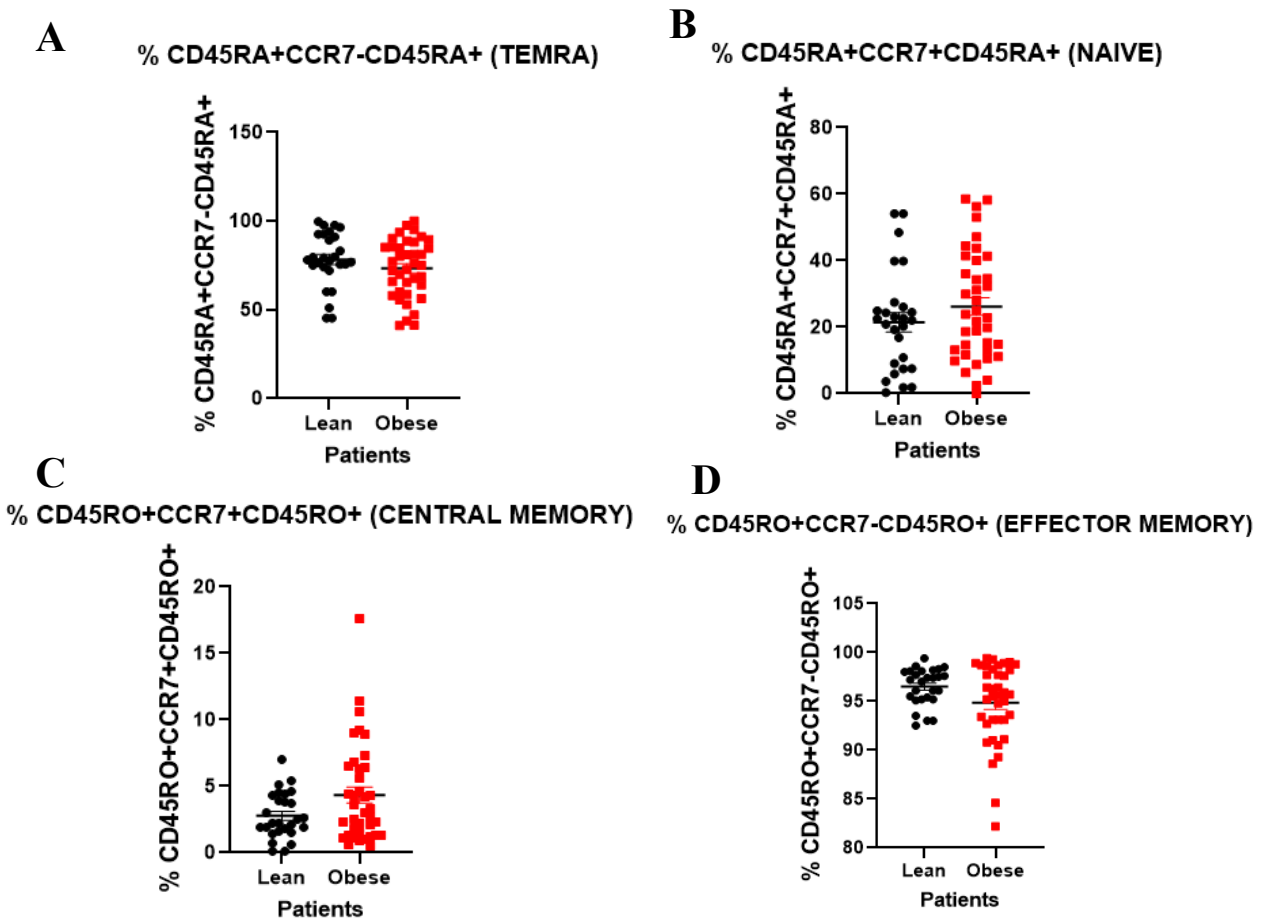


***Figure 36*** *Differences of percentages of numbers in different categories of $CD4^+$ T cells regarding their distribution to $CD45RA^+$ or $CD45RO^+$ markers between lean and obese participants A. $CD45RA^+CCR7^-CD45RA^+$ (TEMRA) T cells (p-value=0.2186, B. $CD45RA^+CCR7^+CD45RA^+$ T cells (NAÏVE) (p-value=0.2556), C. $CD45RO^+CCR7^+CD45RO^+$ (Central Memory) (p-value=0.2086) and D. $CD45RO^+CCR7^-CD45RO^+$ (Effector Memory) T cells (p-value=0.3155). All the values are not statistically significant ($p<0.05$).*

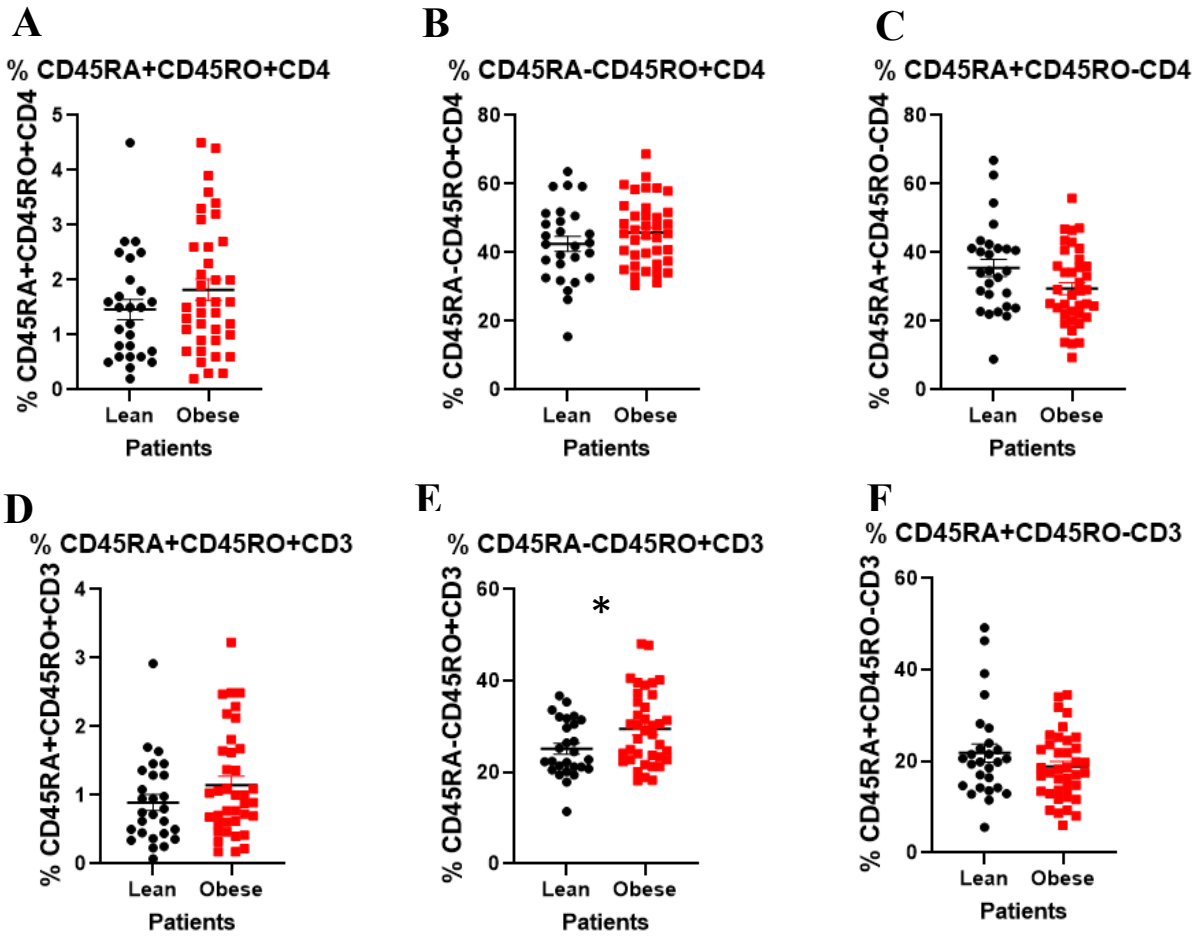


***Figure 37*** *Differences of percentages of numbers in different categories of $CD4^+$ T cells regarding their distribution to $CD4^+$ or $CD3^+$ markers between lean and obese participants A. $CD45RA^+CD45RO^+CD4$ T cells (p-value=0.2063), B. $CD45RA^-CD45RO^+CD4$ T cells (p-value=0.0841), C. $CD45RA^+CD45RO^-CD4$ T cells (p-value=0.3029), D. $CD45RA^+CD45RO^+CD3$ T cells (p-value=0.2552), E. $CD45RA^-CD45RO^+CD3$ T cells (*p-value=0.0391<0.05) and F $CD45RA^+CD45RO^-CD3$ T cells (p-value=0.3522).*

When cell levels were examined regarding their distribution in $CD4^+$ and $CD3^+$ T cell compartment, increased levels of $CD45RA^+CD45RO^+CD4^+$ and $CD45RA^-CD45RO^+CD4^+$ **(Figure 37A, B)** and decreased levels of $CD45RA^+CD45RO^-CD4^+$ **(Figure 37C)** T cells are observed in obese compared to lean individuals. As far as for $CD3^+$ T cells are gated, $CD45RA^+CD45RO^+CD3^+$ T cells have increased levels in obese patients, $CD45RA^-CD45RO^+CD3^+$ T cells are statistically significant elevated in obese ones **(Figure 37D, E)**, while in $CD45RA^+CD45RO^-CD3^+$ T cells decreased levels are observed in obese compared to non-obese individuals **(Figure 37F)**.

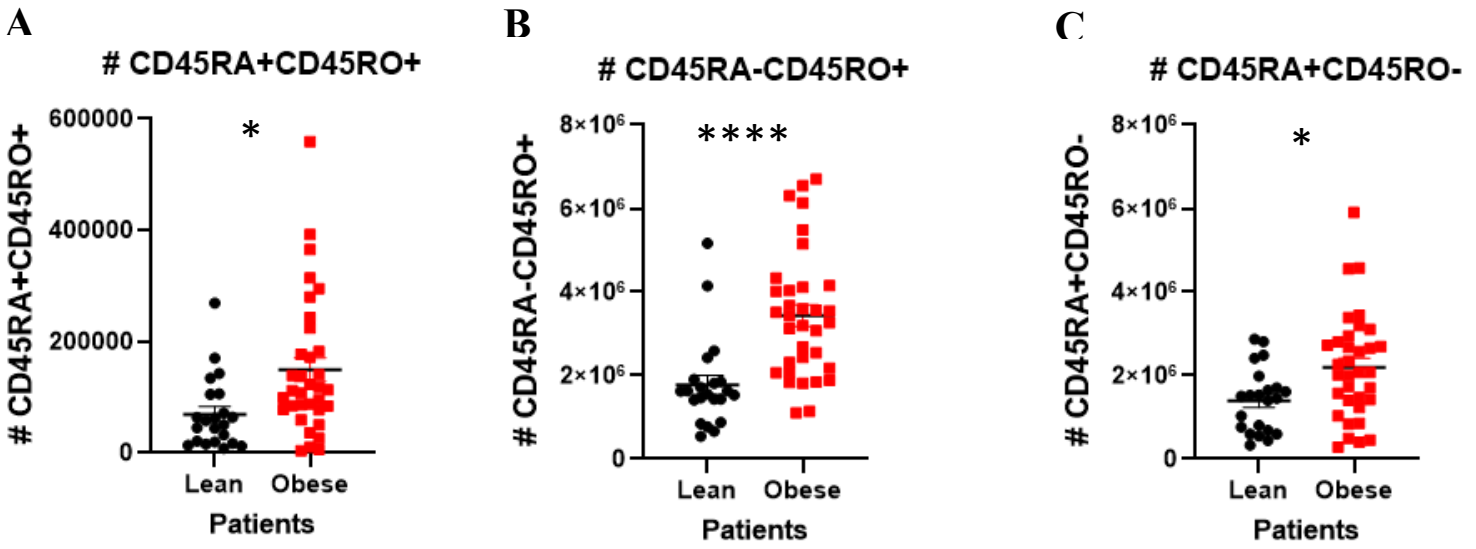


***Figure 38*** *Differences of absolute numbers in different categories of CD4$^+$ T cells between lean and obese participants A. CD45RA$^+$CD45RO$^+$ T cells (*p-value=0.0027), B. CD45RA$^-$CD45RO$^+$ T cells (****p-value=<0.0001), C. CD45RA$^+$CD45RO$^-$ T cells (*p-value=0.0133). All the values are statistically significant (p<0.05).*

Absolute numbers of CD45RA$^+$CD45RO$^+$ T cells (mean±SEM=69421±13625 vs. 149416±21114), CD45RA$^-$CD45RO$^+$ T cells (mean±SEM=1771093±229070 vs. 3432941±259716) and CD45RA$^+$CD45RO$^-$ T cells (mean±SEM=1395053±163826 vs. 2199500±217679) are statistically significant elevated in obese participants compared to lean ones **(Figure 38)**.

The expression of the CXCR3 marker by CD45RA$^+$ and CD45RO$^+$ T cells is also evaluated regarding their distribution in different markers. Absolute numbers of CD45RA$^+$CXCR3$^+$ T cells are statistically significant elevated in obese compared to lean individuals (mean±SEM=47883±9518 vs. 73824±7525) **(Figure 39A)**. Total CD45RA$^+$CXCR3$^+$CD4$^+$ and CD45RA$^+$CXCR3$^+$CD3$^+$ T cells are decreased in obese participants **(Figure 39C, D)**, whereas CD45RA$^+$CXCR3$^+$CD45RA$^+$ T cells have a slight increase in obese patients **(Figure 39B)**.

As far as CD45RO$^+$ T cells are concerned, absolute numbers of CD45RO$^+$CXCR3$^+$ T cells are statistically significant elevated in obese compared to lean individuals (mean±SEM=811460±115362 vs. 1226765±111462) **(Figure 40A)**. Also, total CD45RO$^+$CXCR3$^+$CD45RO$^+$ T cells are statistically significant elevated in obese patients **(Figure 40B)**. Absolute numbers of CD45RO$^+$CXCR3$^+$CD3$^+$ T cells (mean±SEM=7025386±762686 vs. 11986176±818898) are statistically significant

elevated in obese people compared to non-obese **(Figure 41A)**, while their total percentages do not present a notable difference **(Figure 41B)**. Lastly, $CD45RA^{+}CXCR3^{+}CD4^{+}$ T cells are statistically significant elevated in absolute numbers (mean±SEM=4223305±483392 vs. 7598235±542046) and generally increased in total percentages in obese group **(Figure 41C, D)**.

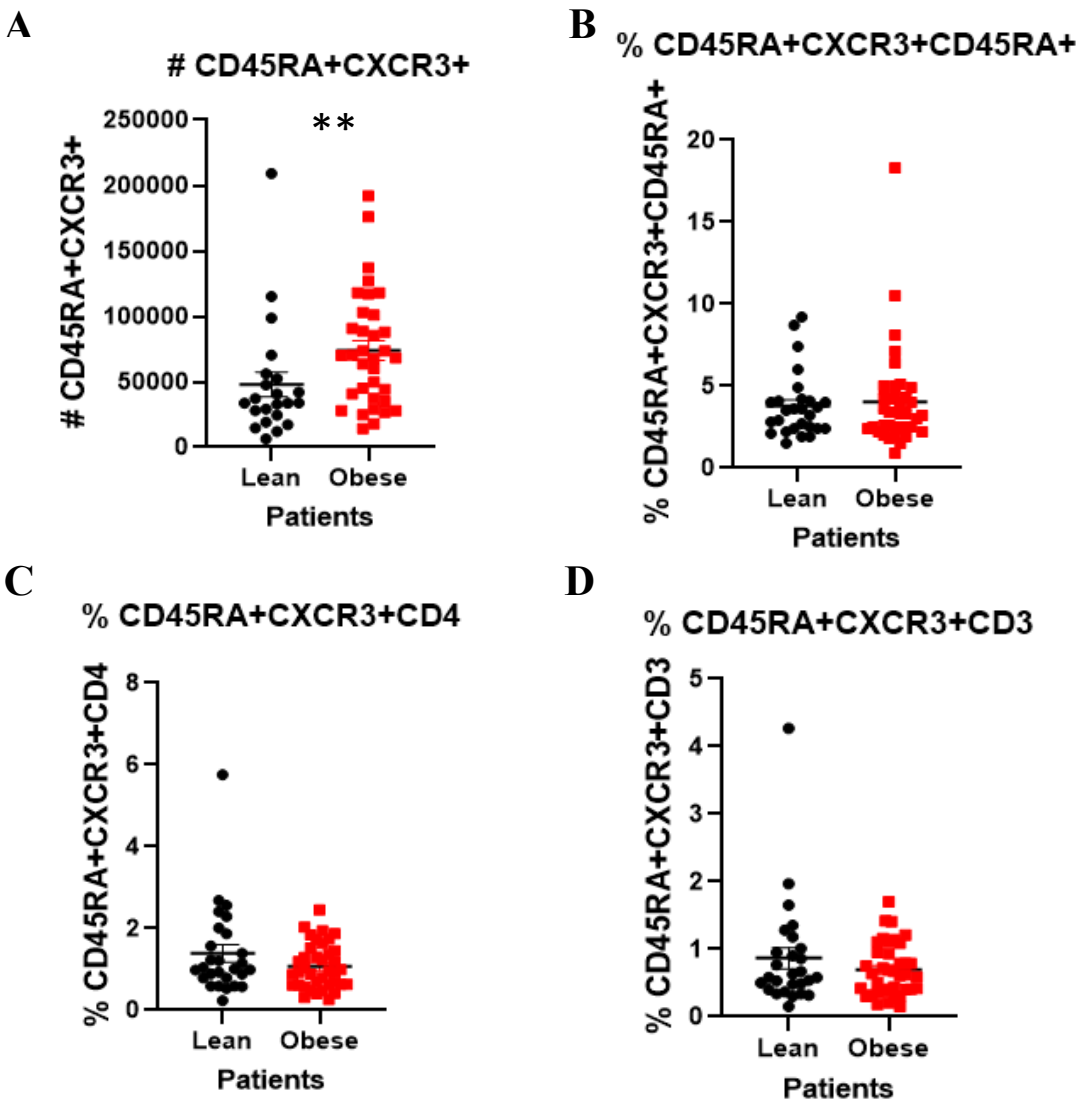


***Figure 39*** *Differences of absolute numbers and percentages in $CD45RA^{+}CXCR3^{+}$ T cells that are gated for different markers between lean and obese participants A. absolute numbers of $CD45RA^{+}CXCR3^{+}$ T cells (**p-value=0.0072<0.05), B. $CD45RA^{+}CXCR3^{+}CD45RA^{+}$ T cells (p-value=0.9710), C. $CD45RA^{+}CXCR3^{+}CD4$ T cells (p-value=0.3487) and D. $CD45RA^{+}CXCR3^{+}CD3$ T cells (p-value=0.6746).*

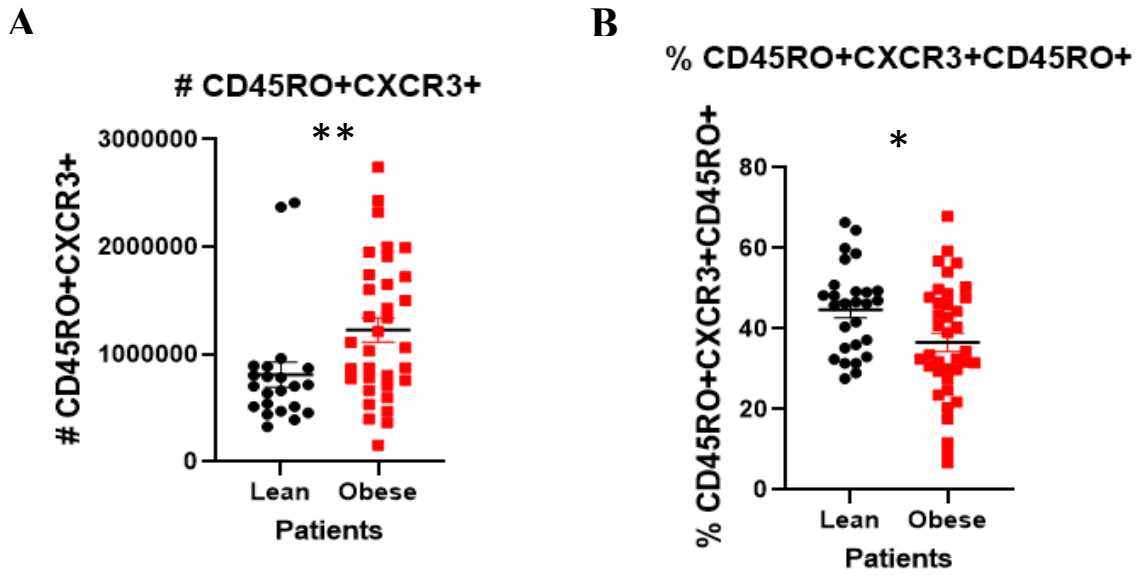


***Figure 40*** *Differences of absolute numbers and percentages in CD45RO$^+$CXCR3$^+$ T cells that are gated for CD45RO$^+$ between lean and obese participants A. absolute numbers of CD45RO$^+$CXCR3$^+$ T cells (**p-value=0.0064<0.05) and B. CD45RO$^+$CXCR3$^+$CD45RO$^+$ T cells (*p-value=0.0193). All values are statistically significant (p<0.05).*

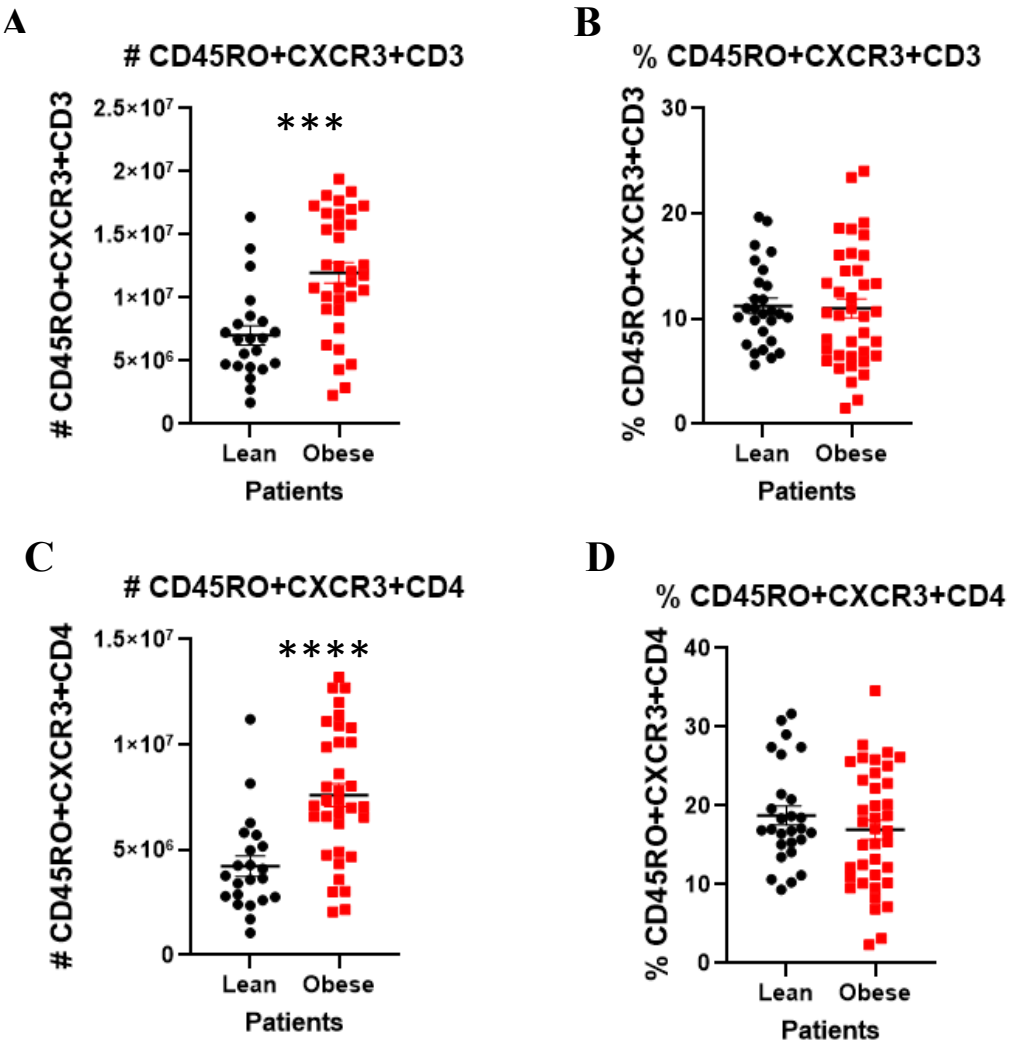


***Figure 41*** *Differences of absolute numbers and percentages in CD45RO$^+$CXCR3$^+$ T cells that are gated for different markers between lean and obese participants A. absolute numbers of CD45RO$^+$CXCR3$^+$CD3 T cells (***p-value=0.0001<0.05), B. CD45RO$^+$CXCR3$^+$CD3 T cells (p-value=0.6177), C. CD45RO$^+$CXCR3$^+$CD4 T cells (****p-value<0.0001) and D. CD45RO$^+$CXCR3$^+$CD4 T cells (p-value=0.3352).*

**B cell compartment**

B cells are found to be elevated in obese compared to lean individuals (in absolute numbers there is statistical significance) (mean±SEM=4454±922.3 vs. 9679±1393) **(Figure 42A, B)**. Regarding their distribution in $CD45^+$ and Not NK ($CD56^-$), $CD14^-CD16^-HLADR^+CD45^+$, $CD14^-CD16^-HLADR^+$Not Neutrophil and $CD14^-CD16^-HLADR^+$Not NK cells are slightly elevated in obese compared to non-obese individuals **(Figure 42 C, D, E)**.

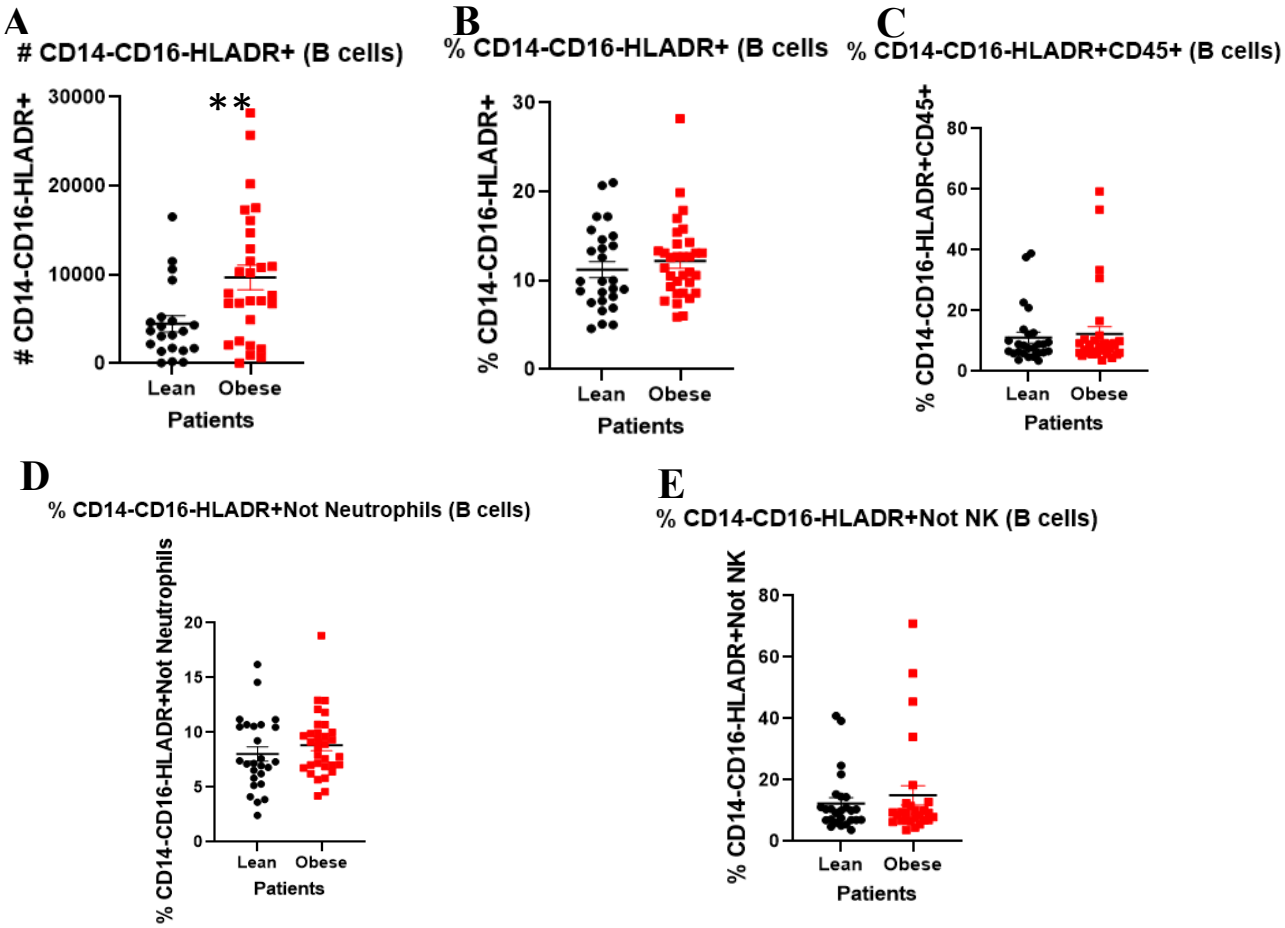


***Figure 42*** *Differences of absolute numbers and percentages in $CD14^-CD16^-HLADR^+$ B cells that are gated for different markers between lean and obese participants A. absolute numbers of $CD14^-CD16^-HLADR^+$ B cells (**p-value=0.0067<0.05), B. $CD14^-CD16^-HLADR^+$ B cells (p-value=0.4441), C. $CD14^-CD16^-HLADR^+CD45^+$B cells (p-value=0.9598), D. $CD14^-CD16^-HLADR^+$Not Neutrophil B cells (p-value=0.3102) and E. $CD14^-CD16^-HLADR^+$Not NK B cells (p-value=0.8083).*

**Natural Killer (NK) cell compartment**

Absolute numbers of NK cells ($CD56^+$ and $CD56^+CD16^+$) are elevated in obese compared to lean individuals (statistically significant in $CD56^+CD16^+$) (mean± SEM=5988±1649 vs. 10149±1663 and mean±SEM=3263±485.6 vs. 6819±1115) **(Figure 43A, B)**. Total percentages of $CD56^+CD45^+$ and $CD56^+CD16^+CD45^+$ NK cells are found to be slightly elevated in obese patients **(Figure 43C, E)**, while percentages of $CD56^+CD16^+CD56^+$ NK cells are decreased in obese compared to lean individuals **(Figure 43D)**.

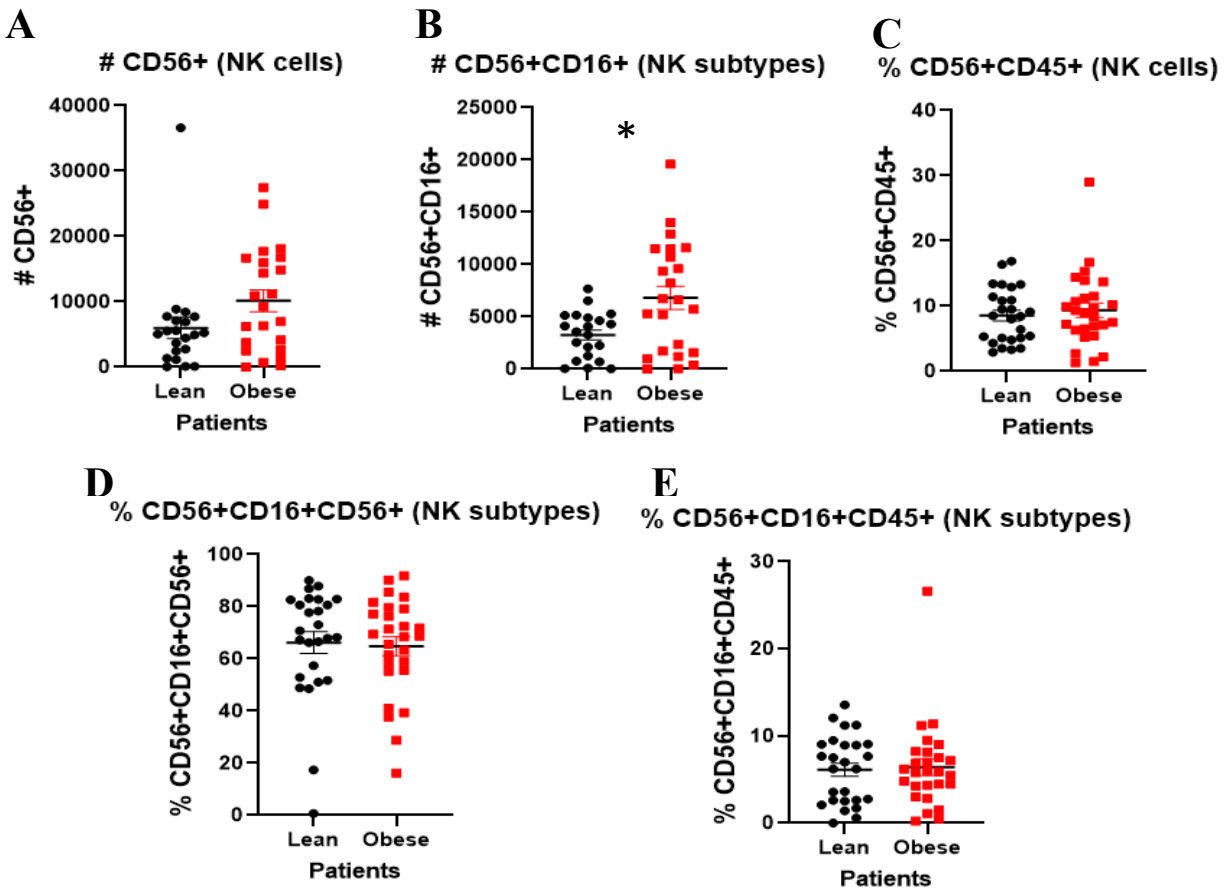


***Figure 43*** *Differences of absolute numbers and percentages in NK cells and their subcategories between lean and obese participants A. absolute numbers of $CD56^+$ NK cells (p-value=0.0596), B. $CD56^+CD16^+$ NK cells (*p-value=0.0213<0.05), C. $CD56^+CD45^+$NK cells (p-value=0.6431), D. $CD56^+CD16^+CD56^+$ NK cells (p-value=0.6494) and E. $CD56^+CD16^+CD45^+$NK cells (p-value=0.8289).*

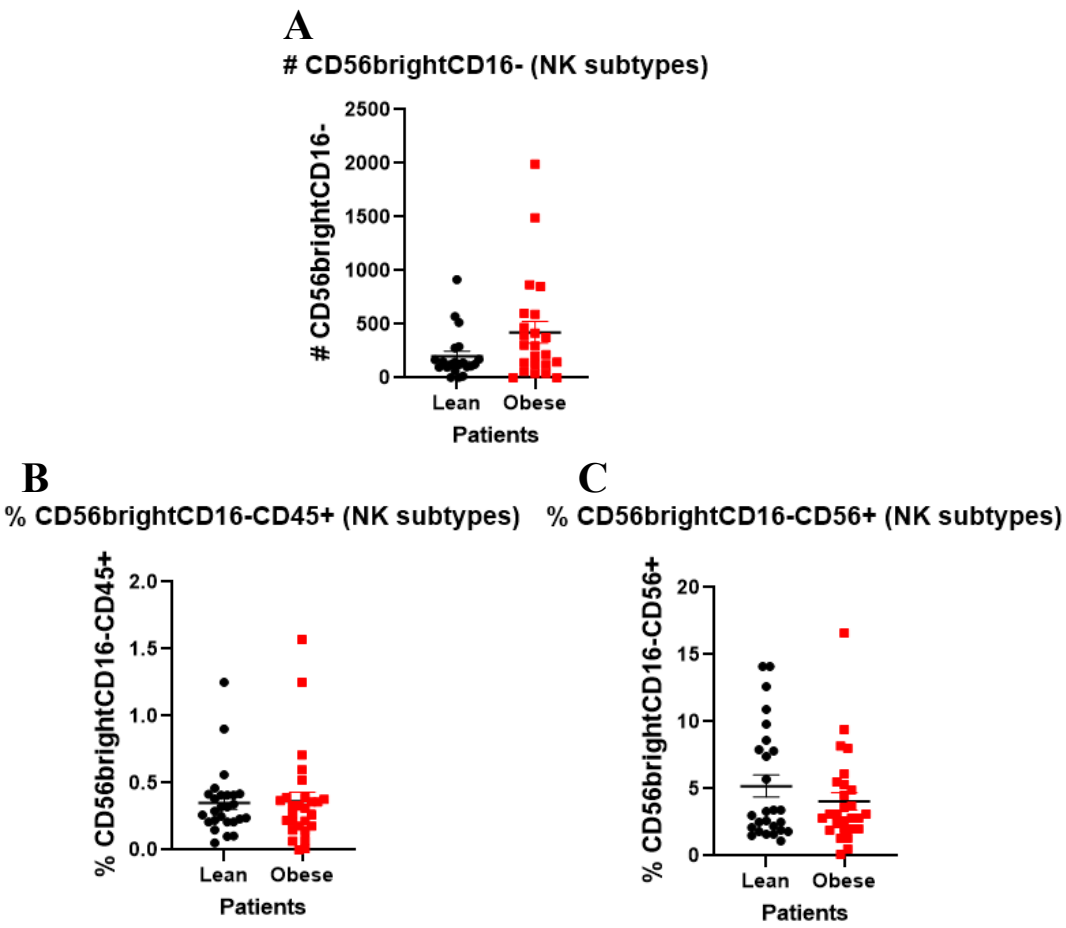


***Figure 44*** *Differences of absolute numbers and percentages in $CD56^{bright}CD16^{-}$ (NK subtypes) cells and their gating for different markers between lean and obese participants A. absolute numbers of $CD56^{bright}CD16^{-}$ NK cells (p-value=0.0831), B. $CD56^{bright}CD16^{-}CD45^{+}$ NK cells (p-value=0.6304) and C. $CD56^{bright}CD16^{-}CD56^{+}$ NK cells (p-value=0.6430).*

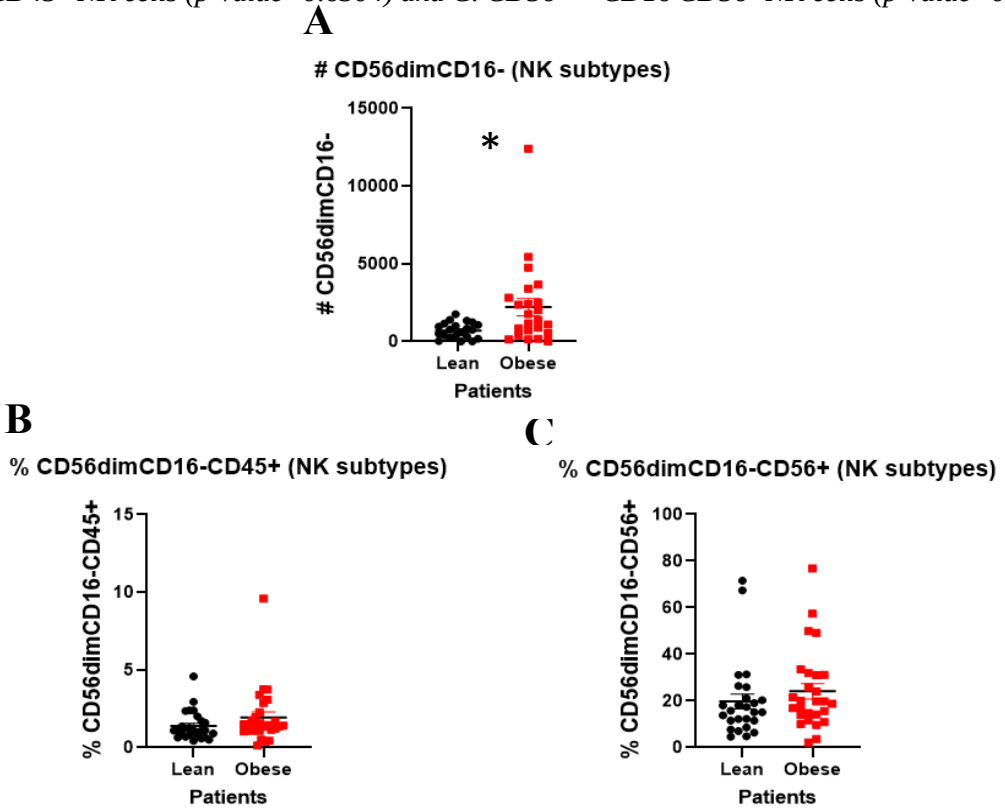


***Figure 45*** *Differences of absolute numbers and percentages in $CD5^{dim}CD16^{-}$ (NK subtypes) cells and their gating for different markers between lean and obese participants A. absolute numbers of $CD56^{dim}CD16^{-}$ NK cells (*p-value=0.0135<0.05), B. $CD56^{dim}CD16^{-}CD45^{+}$ NK cells (p-value=0.1571) and C. $CD56^{dim}CD16^{-}CD56^{+}$ NK cells (p-value=0.2132).*

NK cells can be categorized in two subtypes, $CD56^{bright}CD16^{-}$ and $Cd56^{dim}CD16^{-}$ apart from $CD56^{+}CD16^{+}$. $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{-}$ NK cells are found to be elevated in obese individuals (absolute numbers) (mean±SEM=201.2±47.72 vs. 424.1±120.1 and mean±SEM=709.6±110.3 vs. 2217±557.9) **(Figures 44, 45 A)**. Regarding NK cell distribution, $CD56^{bright}CD16^{-}CD45^{+}$ and $CD56^{dim}CD16^{-}CD45^{+}$ NK cells are found to be increased **(Figures 44, 45 B)**, while there is a reduction in $CD56^{bright}CD16^{-}CD56^{+}$ and an increase in $CD56^{dim}CD16^{-}CD56^{+}$ NK cells in obese compared to non-obese participants **(Figures 44, 45 C)**.

## 3.4. IL-6 in serum samples

Human IL-6 ELISA from R&D systems was used in order to measure the concentration of IL-6 in each sample. Due to the existence of a variety of samples, in one serum sample from lean particpants and in five serum samples from obese ones IL-6 concentration were not measured.

Standard curve was needed in order to find the concentration of IL-6 of each sample based on the absorption values that arised **(Figure 46)**. For this purpose, standard samples with known concentrations of IL-6 have been used **(Table 4)**.

***Table 5*** *Known IL-6 concentrations of standard samples and the correspondence with their absorbance values in order standard curve to be created.*

| **IL-6 Concentration (pg/ml)** | **Absorbance values (Average)** |
|---|---|
| 100 | 1,034 |
| 50 | 0,44055 |
| 25 | 0,27315 |
| 12,5 | 0,1511 |
| 6,25 | 0,0867 |
| 3,13 | 0,06315 |
| 0 | 0,0208 |

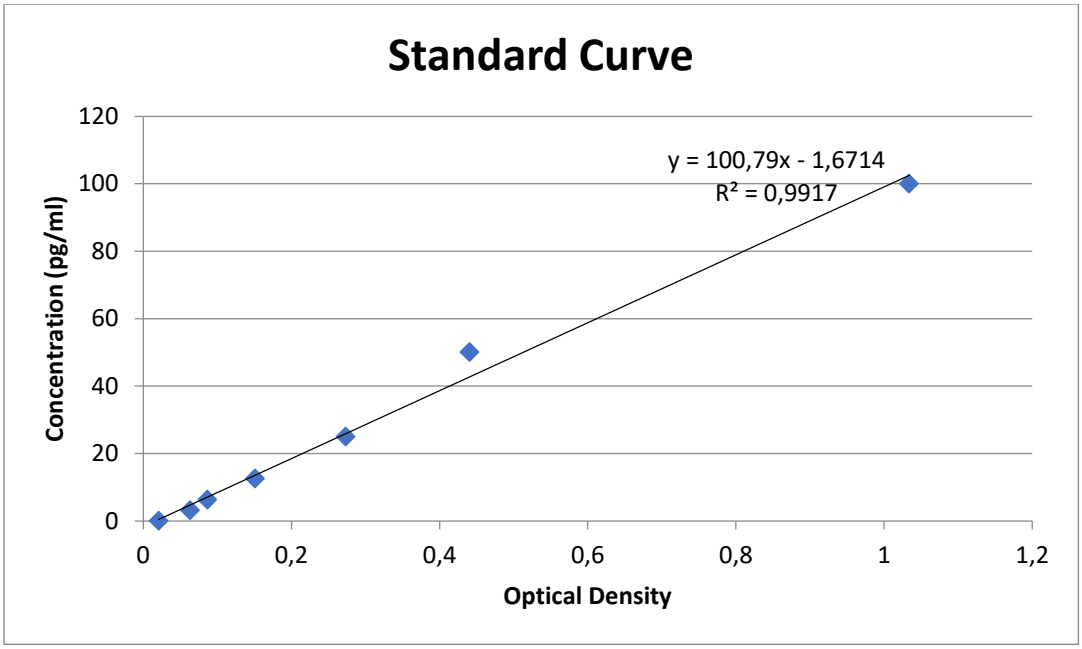


***Figure 46*** *Standard curve of IL-6 for serum samples, where the equation is presented, according to which concentrations of IL-6 of each sample will be calculated.*

Based on the equation of standard curve, IL-6 concentrations of each serum sample were measured. These concentrations were correlated with different characteristics, such as BMI, sex, age, coffee and alcohol consumption, smoking and physical activity (through Mann-Whitney t-test).

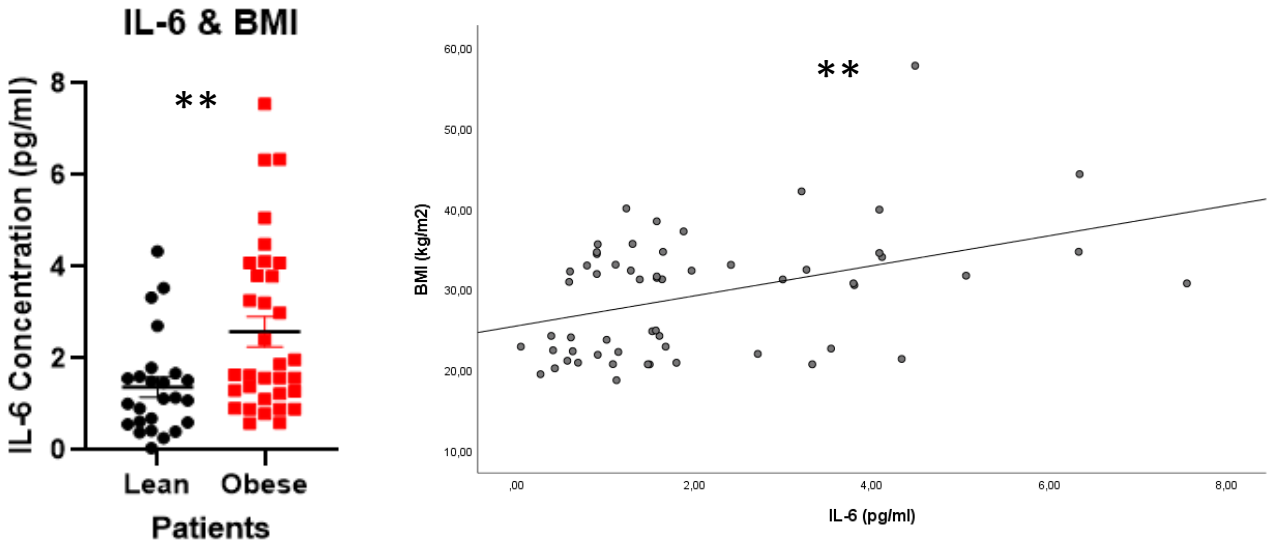


***Figure 47*** *Correlation of IL-6 concentration with BMI of participants A. two categories: lean & obese (**p-value=0.044<0.05)(GraphPad Prism 8) B. Sperman's correlation (IBM SPSS) (**p-value=0.007<0.01)*

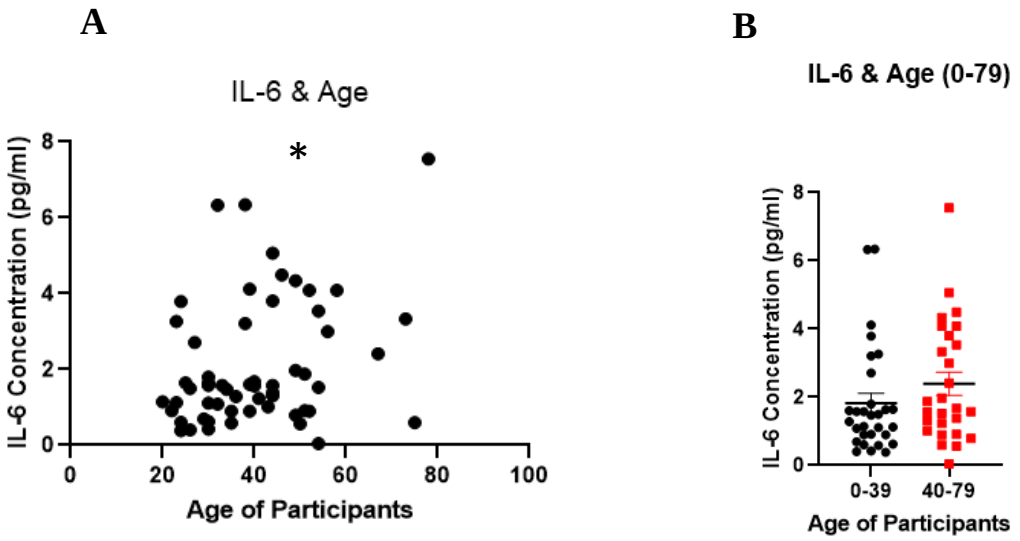


***Figure 48*** *Correlation of IL-6 concentration with A. the age of participants (*p-value=0.0201<0.05), B. two subcategories of participants' age (p-value=0.1327>0.05).*

Based on the above results, there is a statistically significant increase of IL-6 concentrations in obese patients compared to lean subjects (mean±SEM=1.372±0.2165 vs. 2.583±0.3234) resulting in the existence of inflammation with the elevated levels of BMI. This is observed both with Mann-Whitney t-test (p-value=0.044<0.05) and with Sperman's correlation with correlation coefficience to be 0.351 (p-value=0.007<0.01), meaning that it is statistically significant **(Figure 47)**. Also, there is a correlation between the concentrations of IL-6 and the age of participants either generally or by dividing the age in two large subcategories (0-39 years and 40-79 years) (mean±SEM=1.824±0.2871 vs. 2.392±0.3371), meaning that the older a person is, the more IL-6 concentration he has **(Figure 48)**.

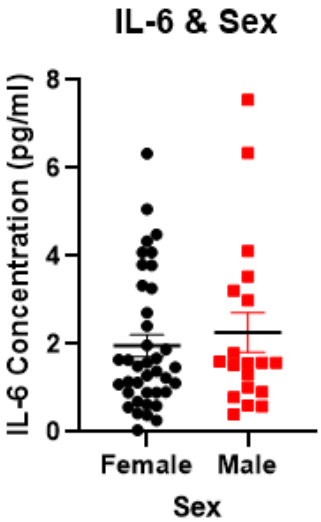


***Figure 49*** *Correlation of IL-6 concentration with the sex of participants (p-value=0.6603>0.05).*

Oly a slight association of IL-6 concentrations in the serum samples with the sex of participants (female-male) was found (mean±SEM=1.963±0.2451 vs. 2.263±0.4499) **(Figure 49)**. One possible explanation could be the larger number of females than males in the study (44 females-20 males).

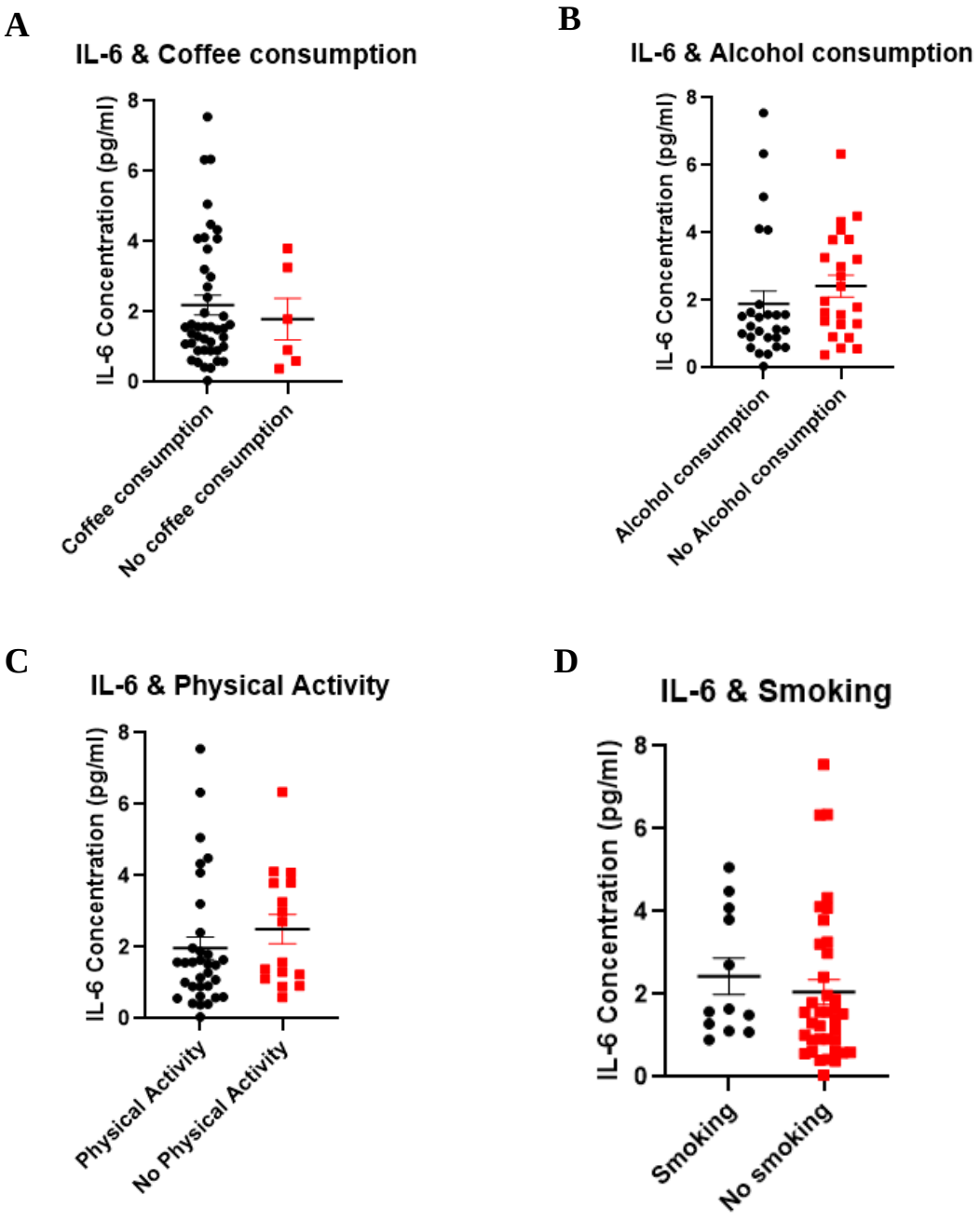


***Figure 50*** *Correlation of IL-6 concentration with A. coffee consumption (p-value=0.6058), B. alcohol consumption (p-value=0.1017), C. physical activity (p-value=0.2207) and D. smoking (p-value=0.2367). Threshold for statistically significant results is p-value<0.05.*

In **Figure 50** correlations of IL-6 concentrations with some variables are presented. More specifically, coffee consumption is related with a slight increase in IL-6 levels in human samples (mean±SEM=2.194±0.2741 vs. 1.793±0.5878) despite the fact that no correlation was found between portions of coffee that are consumed weekly and levels of IL-6. Alcohol consumption is associated with a reduction of IL-6 concentrations (mean±SEM=1.899±0.3741 vs. 2.422±0.3225), but the difference with non-alcohol consumption is not notably important. Physical activity is associated with decreased levels of IL-6 compared with non-exercise (mean±SEM=1.969±0.3137 vs. 2.507±0.4078), while smoking is an aggravating factor as it is related to elevated IL-6 concentrations in serum samples (mean±SEM=2.436±0.4378 vs. 2.050±0.3005). It is worth noting that any of these results are not statistically significant.

When the data of the correlation between BMI and IL-6 are controlled for portions of coffee consumption, correlation coefficient is slightly increased (0.435) (p-value=0.002<0.01), meaning that there is no change in the association of BMI with IL-6 concentrations when a person consumes coffee **(Figure 51)**. When these data are controlled for hours of physical activity, correlation coefficient is also slightly increased (0.401) (p-value=0.005<0.01) compared to the data of the correlation between BMI and IL-6 **(Figure 52)**. Proportional results arise when the data of the correlation between BMI and IL-6 are controlled together for portions of coffee consumption and hours of physical activity, where correlation coefficient is slightly increased (0.416) (p-value=0.004<0.01), meaning that there is no notable change in the association of BMI with IL-6 concentrations when a person consumes coffee and exercise **(Figure 53)**.

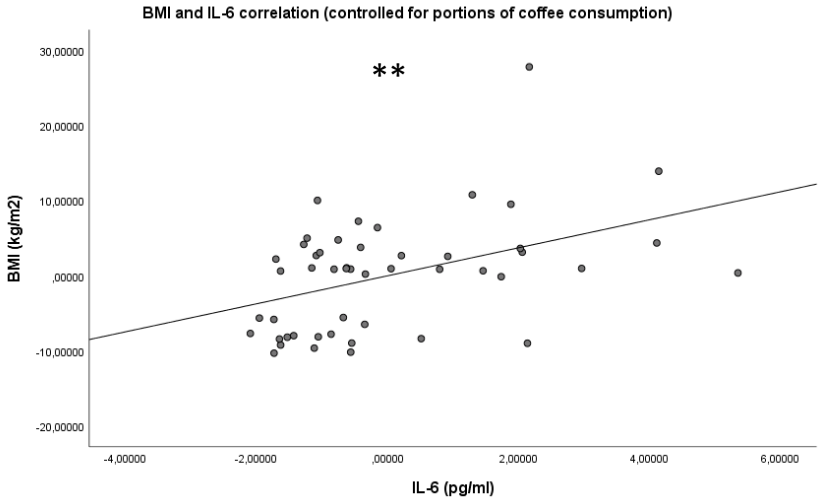


***Figure 51*** *Control of the data of the correlation between BMI and IL-6 for portions of coffee consumption with correlation coefficient=0.435 (p-value=0.002<0.01).*

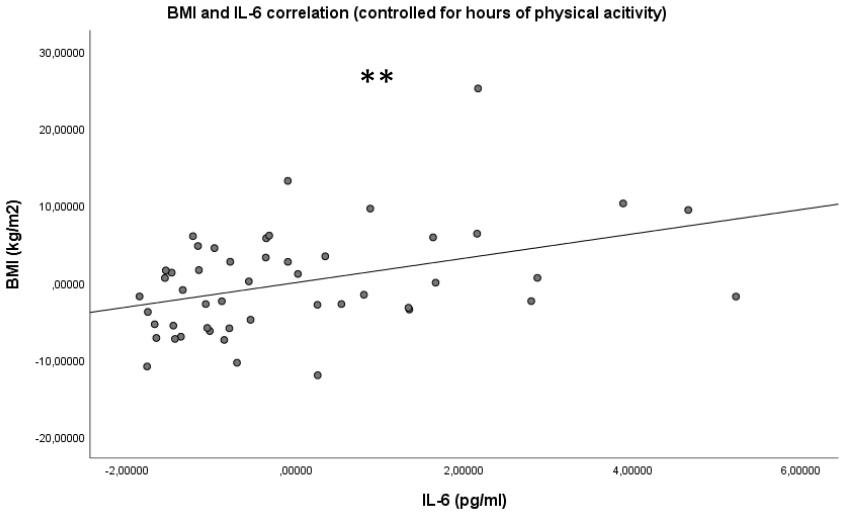


***Figure 52*** *Control of the data of the correlation between BMI and IL-6 for hours of physical activity with correlation coefficient=0.401 (p-value=0.005<0.01).*

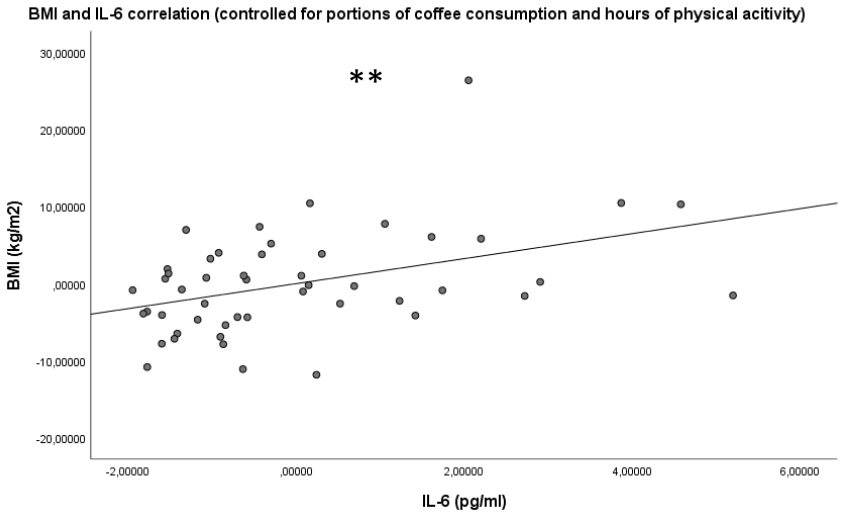


***Figure 53*** *Control of the data of the correlation between BMI and IL-6 together for portions of coffee consumption and hours of physical activity with correlation coefficient=0.416 (p-value=0.004<0.01).*

Lastly, age is a deterrent factor for the reduction of IL-6 levels as the older a person is and the higher BMI has, the levels of IL-6 will be maintained paticularly high without obvious margins for improvement.

## 3.5. IL-6 Concentrations in samples with the induction of B, BL (LPS) media

Human IL-6 ELISA from Origene was used in order to measure the concentration of IL-6 in samples that were induced either with B medium or BL medium (with LPS polysaccharide). Due to the existence of a huge amount of samples, samples only from women were used in this experiment and more specifically, 12 samples from lean participants and 18 samples from obese ones.

Standard curve was needed in order to find the concentration of IL-6 of each sample based on the absorption values that arised **(Figure 54)**. For this purpose, standard samples with known concentrations of IL-6 have been used **(Table 5)**.

***Table 5*** *Known IL-6 concentrations of standard samples and the correspondence with their absorbance values in order standard curve to be created.*

| **IL-6 Concentration (pg/ml)** | **Absorbance values (Average)** |
|---|---|
| 50 | 0,16445 |
| 25 | 0,0905 |
| 12,5 | 0,0434 |
| 6,25 | 0,0248 |
| 3,125 | 0,01765 |
| 1,56 | 0,0123 |
| 0 | 0,0074 |

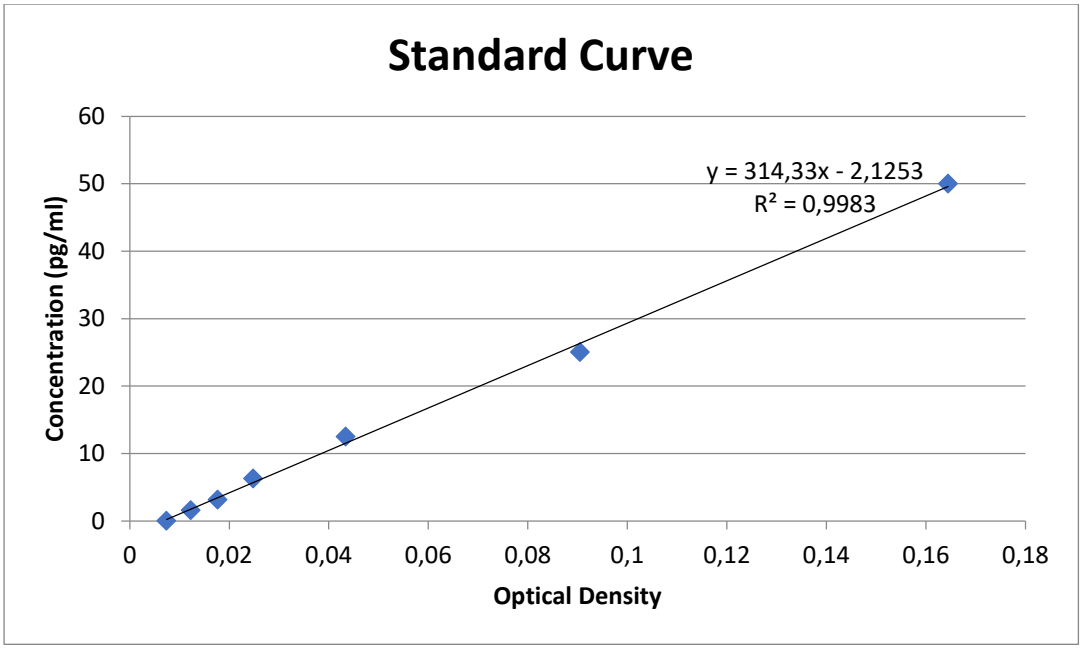


***Figure 54*** *Standard curve of IL-6 for samples with B or BL medium, where the equation is presented, according to which concentrations of IL-6 of each sample will be calculated.*

Based on the equation of standard curve, IL-6 concentrations of each sample were measured. These concentrations were correlated with each other for comparison of the differences that arise after the induction of LPS and with IL-6 concentrations of serum samples in order to find a possible association.

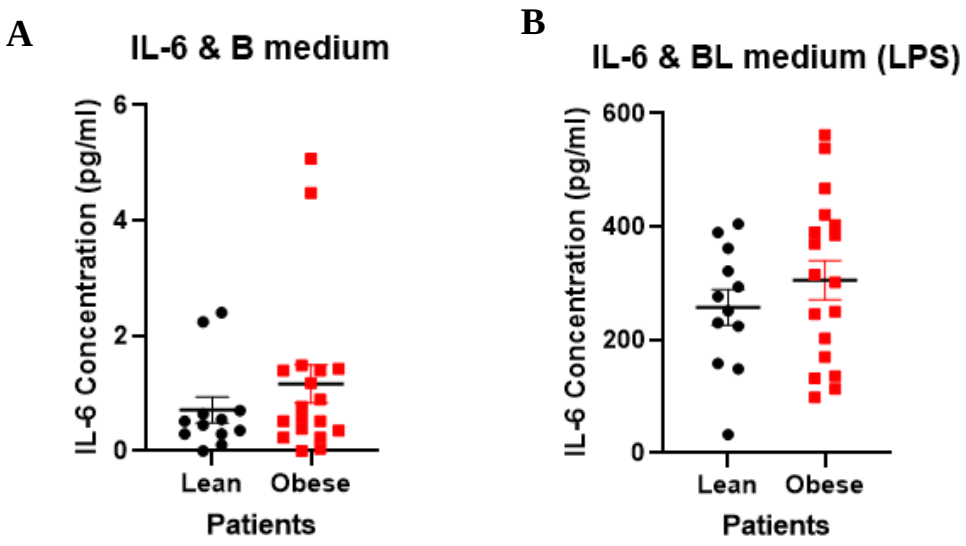


***Figure 55*** *Correlation of IL-6 concentration of samples either after culture A. with B medium (p-value=0.3624>0.05) or B. with BL medium (LPS) (p-value=0.4648>0.05) with BMI (two categories: lean & obese).*

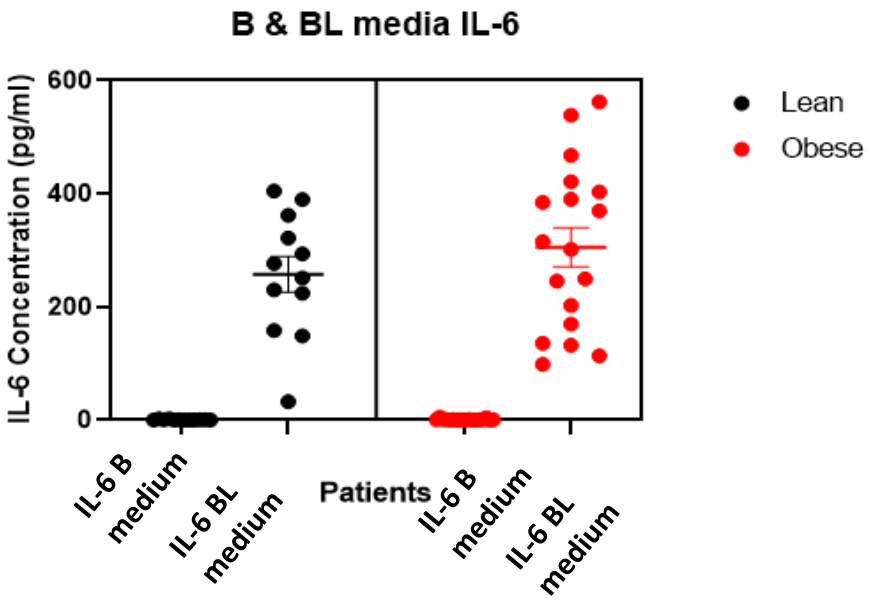


***Figure 56*** *Comparison of IL-6 concentrations between samples from lean and obese patients that are induced eithet on B medium or BL medium (LPS) (p-values>0.05).*

It is observed that in both cases meaning in samples that are induced either with B medium or with BL medium (contains LPS for extra induction of obesity), IL-6 concentration is elevated in obese patients (mean±SEM=0.7131±0.2250 vs. 1.164±0.3311 in B medium) (mean±SEM=257.9±31.38 vs. 305.7±34.37 in BL medium). Especially, in case of BL medium both lean and obese subjects perform huge IL-6 levels (large differentiation from samples that are cultured with B medium). In other words, BL medium causes a huge increase in IL-6 levels that does not differ notably between the two subcategories, lean and obese (not statistically important results) **(Figures 55 & 56)**.

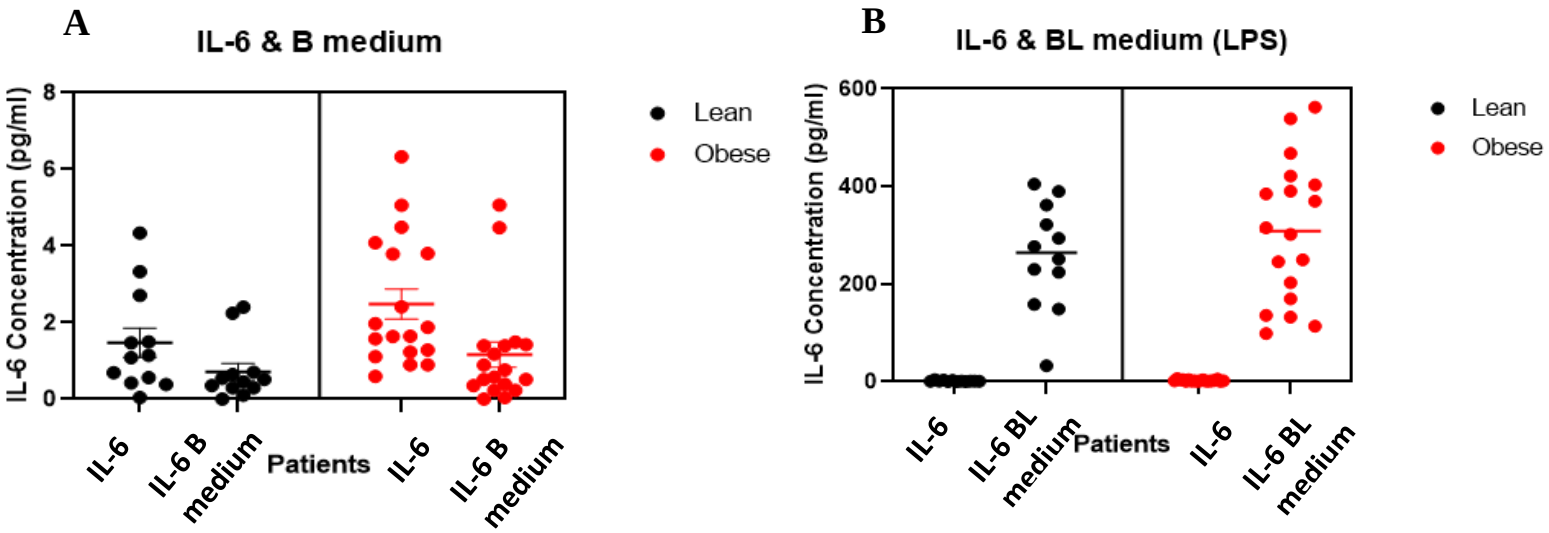


***Figure 57*** *A. Comparison of IL-6 concentrations between samples from lean and obese subjects that are derived from serum or from cells that are cultured by B medium. B. Comparison of IL-6 concentrations between samples from lean and obese subjects that are derived from serum or from cells that are cultured by BL medium (p-values>0.05).*

It has been found that in both lean and obese participants IL-6 concentrations are slightly decreased when cells are cultured in B medium compared with serum samples, whereas exactly the opposite is observed in the case of BL medium meaning that there is a huge increase in IL-6 levels in samples that are induced by BL medium both at lean and obese subjects compared with IL-6 levels in serum samples **(Figure 57)**.

## 3.6. Genetic Risk Scores for obesity

Genetic Risk Score (GRS) for Obesity that has arised from 32 SNPs and 16 rare SNVs together with their correlation and gravity-frequency with BMI (Section "Methods"), gives information about genetic predisposition to obesity. Therefore, the higher the GRS value is, the more likely is a person to be obese or in order to stay lean and healthy, diet (caloric resitriction) and physical exercise are needed.

GRSs for Obesity are correlated with BMI, age and sex and the results are depicted in the following figures (comparisons were performed using Mann-Whitney t-test).

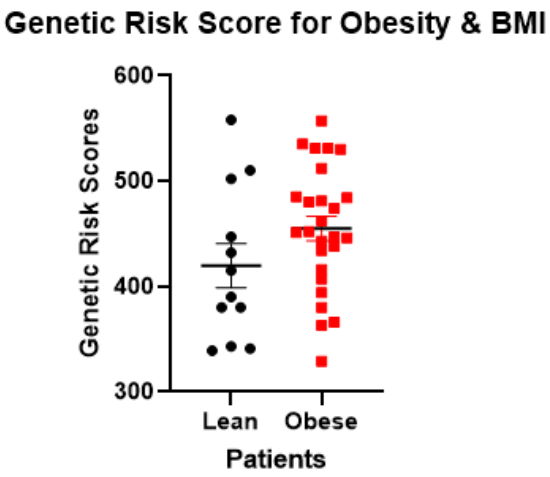


***Figure 58*** *Correlation of GRS for Obesity with BMI of participants (two subcategories: lean and obese) (p-value=0.1081>0.05).*

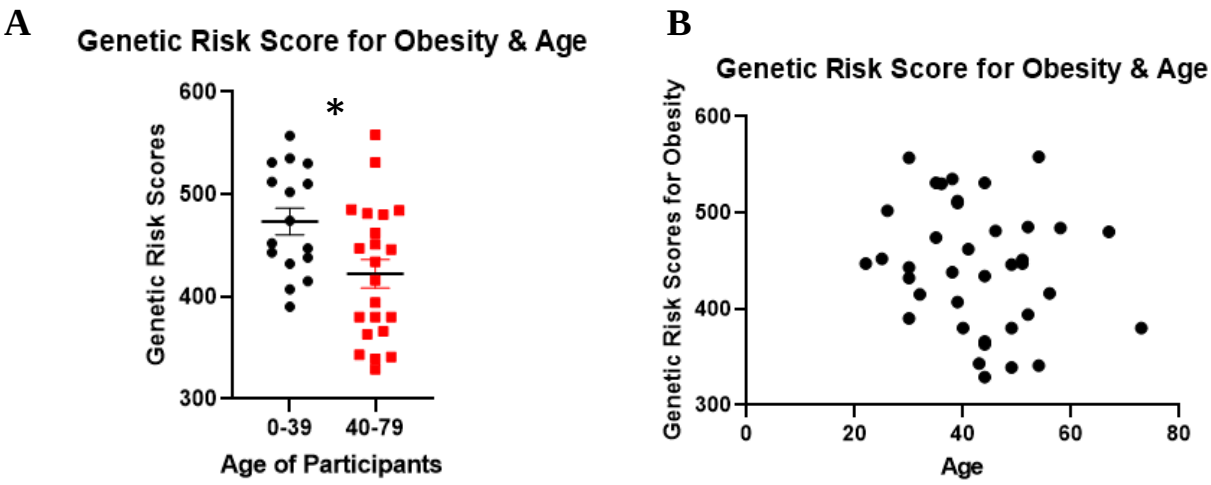


***Figure 59*** *Correlation of GRS for Obesity with A. two subclasses that age of participants is divided (*p-value=0.0258<0.05) and B. the age of participants (p-value=0.2215>0.05).*

As it was expected, BMI is correlated with genetic risk score for obesity. In other words, obese people are related to higher genetic risk scores than lean ones (mean±SEM=419.8±20.89 vs. 454.9±11.58) **(Figure 58)**. On the other hand, GRS is higher in younger participants (0-39 years) compared with older ones (40-79 years) (mean±SEM=473.4±13.03 vs. 422.3±13.95), while no specific association is shown when age is not subdivided in categories **(Figure 59)**.

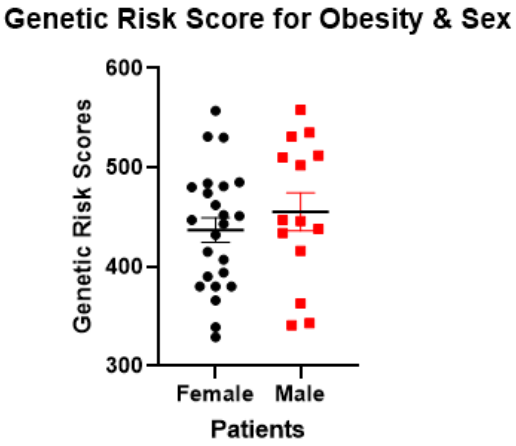


***Figure 60*** *Correlation of GRS for Obesity with sex of participants (p-value=0.4229>0.05).*

As far as sex is concerned, an upward trend of genetic risk score in male participants compared to females is observed despite the fact that the result is not statistically sigmificant (mean±SEM=437.0±12.35 vs. 455.4±19.28) **(Figure 60)**.

## 3.7. Genetic Risk Scores for telomere length

Genetic Risk Score for telomere length is a marker for cardiovascular diseases because of the fact that its result is linked to cardiological age. Therefore, the older a person's cardiological age compared with his actual age is, the greater the risk for cardiovascular diseases is. It is worth noting that GRS for telomere length results from the analysis of 7 SNPs as in the Section "Methods" is mentioned.

For this purpose, GRS for telomere length of participants was analyzed taking into account either age or BMI or sex or IL-6 concentrations.

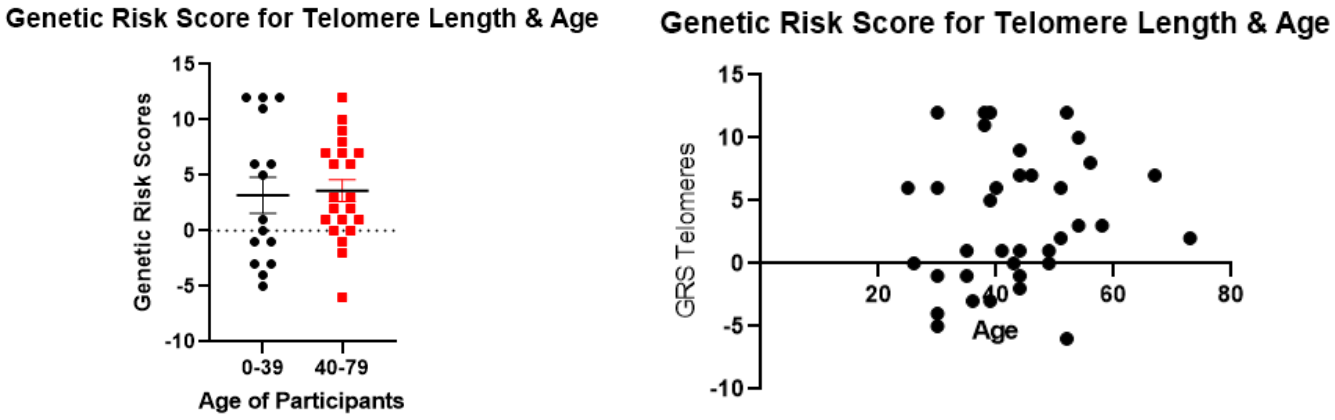


***Figure 61*** *Correlation of GRS for Telomere Length with A. two subclasses that age of participants is divide and B. the age of participants (p-value=0.5616>0.05).*

A slight increase in the older compared to younger participants is observed in GRS for telomere length (with the age of participants either generally or specifically in the two subcategories of 0-39 and 40-79 years) resulting in possible decreased risk for cardiovascular diseases (mean±SEM=3.200±1.631 vs. 3.619±0.9795) **(Figure 61)**.

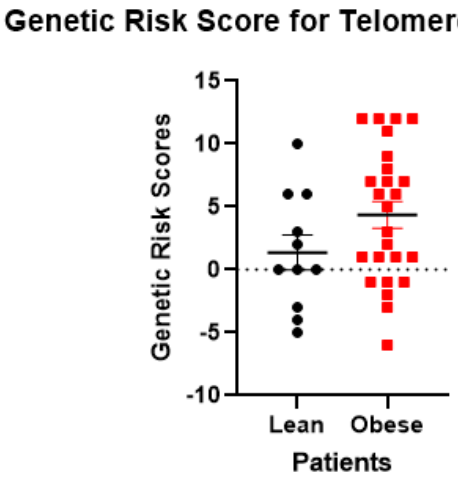


***Figure 62*** *Correlation of GRS for Telomere Length with BMI of participants (two subcategories: lean and obese) (p-value=0.1161>0.05).*

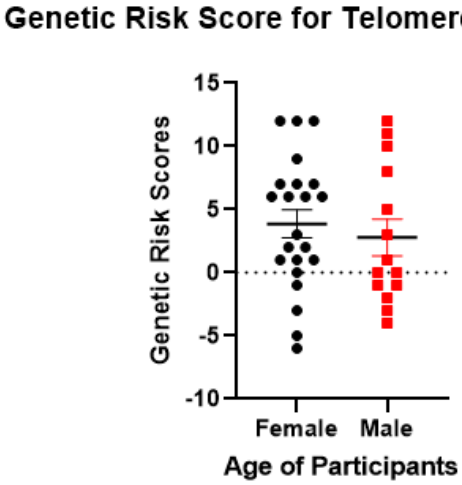


***Figure 63*** *Correlation of GRS for Telomere Length with sex of participants (p-value=0.4070>0.05).*

Despite the fact that there is no statistical significance, notably elevated numbers of genetic risk score for telomere length in obese participants compared to lean ones are observed (mean±SEM=1.364±1.397 vs. 4.360±1.064) **(Figure 62)**. However, as far as sex of participants is concerned, a slightly increase of GRS for telomere length is presented in females (mean±SEM=3.864±1.107 vs. 2.786±1.457) **(Figure 63)**.

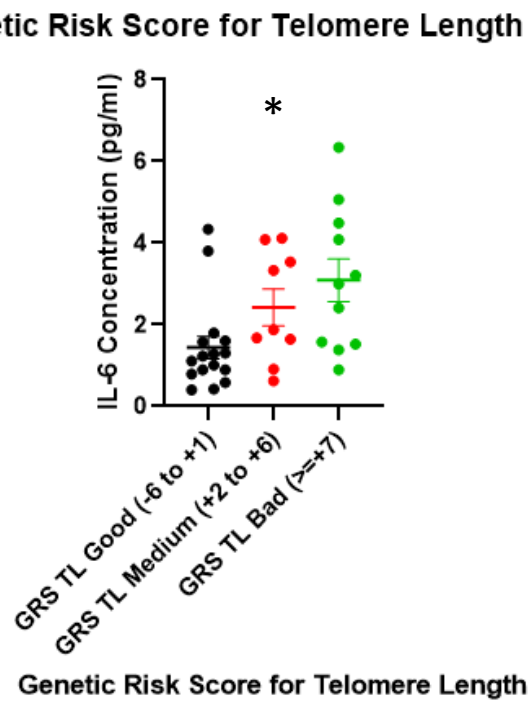


***Figure 64*** *Correlation of IL-6 concentration and Genetic Risk Score for Telomere Length. Values of GRS from -6 to +1 have a good impact in person's health (low risk of cardiovascular diseases), from +2 to +6 are characterized as medium, while values >=+7 are associated with high risk of cardiovascular diseases (*p-value=0.0151<0.05).*

GRS for telomere length can be categorized in subclasses based on the values that arise from the analysis of SNPs. In the first category belong GRSs with values from -6 to +1, meaning that a person with a value -5 has a cardiological age 5 times smaller than his actual age. So, people in this category have a good prognosis for their health status. In the second category that is characterized as medium belong GRSs with values from +2 to +6, whereas GRSs with calues >=+7 belong to the third category, where people have a bad prognosis as far as their cardiological health is concerned.

In **Figure 64** a statistically significant association between pro-inflammatory cytokine IL-6 concentrations and GRS categories is observed. In other words, higher levels of IL-6 concentrations are linked to higher values of genetic risk score for telomere length.

# 4. Discussion

In the present study correlations between blood cells and obesity, pro-inflammatory cytokine IL-6, obesity and other parameters, as well as between genetic risk scores and IL-6 or obesity were investigated. It is well known that obesity is associated with chronic inflammation by producing a variety of cytokines in humans' serum and problems in whole immune system resulting in many co-morbidities (O'Rourke, et al., 2005). For this purpose in this study proinflammatory status of obese people is being examined by different aspects.

Based on literature it seems that white bood cells are correlated positively with BMI, while only few studies support the opposite result maybe due to the limited number of the sample, such as in the study of Tanaka, et al., 2001.

Neutrophils, that constitute the majority of polymorphonuclear leukocytes, based on the results of this study, are found slightly increased in obese participants in comparison with the other two types of leukocytes, basophils and eosinophils that are found to be decreased. This outcome is supported by many studies and can be explained by the fact that neutrophils are the first immune cells resulting in direct inflammation (Xu, et al., 2015). As far as basophils and eosinophils are concerned, this decrease is not confirmed by studies in the literature in which increased levels of all leukocytes are observed (Xu, et al., 2015). Although obesity and both eosinophils and basophils are related to allergic inflammation, this conflicting outcome arises.

PBMCs, that consist of monocytes and lymphocytes, are statistically significant increased in obese participants. As far as monocytes are concerned, according to the above results, all types of monocytes are related to obesity as they have been found increased in obese participants. That is confirmed by a variety of studies (Friedrich, et al., 2019), while some of them have oscillating outcomes reporting no clear association between monocyte levels and BMI (Krinninger, et al., 2014). In another study, non-classical monocytes are the only subcategory of monocytes that is not directly related to obesity as they do not contribute to monocytosis in obesity (Friedrich, et al., 2019). It is known that intermediate and non-classical monocytes are associated with the initiation of immune response in case of an inflammatory situation resulting in a possible upregulation in obesity. This is a possible explanation about the results of this study. Also, a marked increase in $CD14^{low}CD16^{+}$ monocytes is

observed in obese participants compared to lean ones emphasizing the proinflammatory role of these cells in obesity-related systemic inflammation.

Regarding lymphocytes, an increase in their levels is observed in the results of this study that has statistical significance. These are in accordance with the majority of studies in the literature. NK cells ($CD56^+$, as well as two NK subtypes $CD56^{bright}CD16^-$ and $CD56^{dim}CD16^-$) are found to be elevated in obese participants compared to non-obese ones as the majority of blood cells. This result contradicts with the studies found on the literature, where reduction in NK cell ($CD56^+$) total levels is reported in the obese participants as obesity is associated with impaired NK cells (Lynch, et al., 2009). Only in the case of $CD56^+CD16^+CD56^+$ cells, a reduction in their levels is observed in obese compared to lean individuals. Because of the fact that NK cells are involved in immunity and cancer, its role could be very useful for possible predisposition of obesity to higher risk of cancer. However, there are studies that support that NK levels are elevated in obese ones (Magrone, et al., 2017). Therefore, further analysis, experimental research and larger sample number in the studies are needed for clearer and reliable outcomes.

T regulatory cells belong to the category of anti-inflammatory molecules as inflammation that is observed in obese individuals could be due to impaired Tregs function-numbers (Švec, et al., 2007). In this study, Treg cells are elevated in obese participants compared to lean ones. Van Der Weerd, et al., (2012) have found an increase in circulating Treg cell counts in morbidly obese adults and a positive correlation with BMI. On the contrary, Tregs percentage to $CD4^+$ (and $CD3^+$) T cells are decreased in obese compared to non-obese subjects, which is also reported by Wagner, et al., (2013) and Agabiti-Rosei, et al., (2018). So, Tregs could be very useful in immune system and obesity with their anti-inflammatory functions.

B lymphocytes, as well as T cells, are found elevated in obese participants. To this line, obesity is associated with elevated B cell numbers in several studies (Philips, et al., 2010). As far as T cells are concerned, a statistically significant increase in $CD3^+$ T cells is observed in obese participants, which is opposed by Tanaka et al., 2001, where a statistically significant reduction in $CD3^+$ cells was found in obese compared to lean subjects. $CD3^+$ T cells that are gated-classified for $CD4^+$ cells are statistically significant increased in obese individuals, which is confirmed by literature. However,

Tanaka et al. 2001 reported a reduction in $CD3^+CD4^+$ T cells in obese subjects. Furthermore, $CD8^+$ cells are found to be increased in obese people despite the fact that $CD8^+CD3^+$ cells are reduced. This is in accordance with the confusion that exists in literature as in some studies $CD8^+$ are reported either decreased or without difference between obese and non-obese people (O' Rourke, et al., 2005), while in others (Womack, et al., 2007; Magrone, et al., 2017) elevated numbers of $CD8^+$ cells are presented. It is well known that $CD8^+$ cells are less prone to adipokine activation as a result they get activated by $CD4^+$ T cells that use fat-induced adipokine production. As a consequence, fat accumulation is more possible to be found on $CD8^+$ T cells in extreme BMI condition compared to lean state (Womack, et al., 2007).

Central and effector memory T cells are statistically significant increased in the obese group. This is confirmed by the study of Van Der Weerd, et al., 2012, where central memory cells are observed in obese subjects, while it is opposed by Tanaka, et al., 2001, who have observed significantly lower memory T cells in obese than lean control subjects. On the other hand, Mauro, et al., 2017 demonstrated a significant association between increased effector memory T cells and obese state. From literature it is known that memory cells have already been exposed to the antigen and still exist in its absence, while they possess enhanced activity in case of re-exposure and solicit immune response (Rosenblum, et al., 2016) and they have been strongly associated with obese condition (Mauro, et al., 2017). More specifically, central memory T cells that have little or no effector function can proliferate and differentiate into effector cells in response to stimulation by antigens. Effector memory T cells possess immediate effector function. Therefore, memory cells are associated with the accumulation of inflammatory T cells in fat sites during obesity.

Naïve cells are the first stage of differentiation of T cells to $CD45RA^+$ T cells, while TEMRA cells belong to the final stage of this differentiation. Naïve T cells are responsible for coping with pathogens that are not neutralized by immune system and may further differentiate into memory cells. TEMRA T cells are cytolytic cells that have lower homeostatic proliferation capacity and shorter telomeres than naïve, central memory and effector memory T cells. $CD45RA^+$ T (TEMRA and naïve) cells are found elevated in obese individuals in this study and it is confirmed by Van Der Weerd, et al., 2012. Also, $CD45RA^+CD45RO^+CD4^+$cells are found slightly upregulated in obese participants. These cells resemble naïve T cells in the inhibition

of immunoglobulin production by B lymphocytes, resemble memory T cells in the expression of mRNA and the production of IFN-γ and are a transient stage of differentiation in the process of transformation from naïve to memory cells. As a consequence, the overall increase in $CD4^+$ T cells in the obese group could be attributed to the increase reported mainly in central memory T cells and to a lesser extent in naïve and effector memory T cells.

The expression of CCR7 marker is related to the sequential differentiation of $CD4^+$ cells from naïve to TEMRA cells, where central and effector memory T cells belong to the intermediate stages. The expression of CXCR3 marker in obesity is linked to a reduction of naïve/TEMRA cells and an increase of memory cells in obese participants.

Consequently, obesity is directly associated with the regulation of inflammatory and immune responses as it is related to increased frequency of the majority of blood cells (e.g. PBMCs) confirming the proinflammatory status of obese people. It is evident that current literature is characterized by conflicting data regarding most of the cells included in this study. Those variations might be attributed to differences in the BMI of participants or to their possible existing co-morbidities (O'Rourke, et al., 2005). Therefore, well-designed studies with a large number of sample that will check immune cell frequency and function from adipose tissue from the same person is an emergency. In this way, scientists will understand the mechanism behind the association of immune cells with obesity implicating possible treatment for obesity-caused diseases or circulating immune cells might be used as biomarkers.

As far as pro-inflammatory cytokine IL-6 is concerned, it is strongly correlated with BMI. This means that obese people have higher levels of IL-6 causing low grade chronic inflammation as a result many health problems can be arised in the future. These high levels of IL-6 with the increase of numbers of BMI have been prooved in many studies, such as Mendez-Garcia, et al., 2020. This strong correlation can be controlled by other parameters, such as coffee consumption and physical exercise achieving the strengthening of this association. More specifically, coffee consumption is related to slightly higher levels of IL-6, which can be explained by the fact that the metabolic potential of coffee in each person based on genetic data was not taken into account. Also, this result is not statistically significant and might reflect to non-

difference, like the study of Gavrielli, et al., 2011. Paradoxically, alcohol consumption is related to lower levels of IL-6 and inflammation. This is confirmed by the study of Beulens, et al. where it was found that alcohol consumption is linked to lower risk of T2DM despite the fact that no mechanism was found based on adiponectin (Beulens, et al., 2008). Smoking, as it is expected, is related to higher levels of IL-6 without statistical significance. Lastly, physical activity is associated with lower levels of IL-6 and reduction in inflammation, which is confirmed by a variety of studies like Lavoie, et al., 2010. Based on Tay, et al., 2019 chronic inflammation is related to a lower physical activity, while intervention through physical exercise for months in children can lead to decrease of BMI and improvement of inflammation state (Coimbra, et al., 2017). All these values are not statistically significant because of the limited sample number. Age is, also, related to decreased IL-6 levels meaning that the older a person is and the higher BMI has, the levels of IL-6 will be maintained paticularly high.

Cells from serum of samples that are cultured with B medium (RPMI) have higher IL-6 levels in obese compared to lean ones, while the levels of IL-6 in these cells are lower than IL-6 levels that are measured in serum samples both at lean and obese participants maybe due to the nutrients that this medium has. LPS that is added in the medium causes a huge increase in the levels of IL-6 compared to IL-6 that is produced by serum samples and by samples that were cultured in B medium. As it is expected, IL-6 levels in BL medium (LPS) are elevated in obese compared to non-obese participants. This happens because LPS is a stimulant of immune responses that mimicks obesity conditions, while it can induce NFkB and MAPK-dependent proinflammatory cytokine/chemokine expression in preadipocytes (Chung, et al., 2006).

Last but not least, genetic risk score for obesity is associated with elevated numbers of BMI as in some studies it has been found (Speliotes, et al., 2010; Viljakainen, et al., 2019). GRS is a marker that is linked to predisposition to obesity and as a result the higher their levels are, the more likely is a person to become or to be obese having higher levels of BMI. So, these people based on the above outcomes will be characterized by high levels of IL-6 and inflammation that can be reduced by reduction of BMI as IL-6 and BMI are strongly correlated. This can happen not only by caloric restriction (diet), but also by physical activity as it is related with lower

levels of IL-6 and generally an improvement in immune system and health. In addtion, GRS for obesity has been found to decrease as the age passes, as a person gets older. In other words, the older a person, is, the smallest the risk for obesity is. This result that is statistically significant, can be explained by the fact that the sample was not uniform as far as age is concerned.

Genetic Risk Score for telomere length that is a marker for cardiological age has been found to be increased in obese individuals, as BMI increases (Nacopoulos, et al., 2018). This is sensible as when BMI is increased, inflammation is elevated that can lead to inflammaging, which is connected with the increase of age. Therefore, when BMI increases, cardiological age increases and can be notably higher than the actual age. However, an important finding is that GRS for telomere length is directly linked to IL-6 levels. Higher levels of IL-6 concentrations are associated with higher values of genetic risk score for telomere length resulting in higher risk for cardiovascular diseases as cardiological age is increased.

Consequently, inflammation plays an important role in obesity and aging. Apart from genetic characteristics that can lead to bad prognosis both for obesity and cardiovascular diseases, BMI is determinant factor for a person's health. Despite the fact that more research in this field is needed, physical exercise and diet can be possible solutions for reduction of inflammatory factors-inflammation and therefore decrease in different obesity- and age-related diseases.

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