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HUMAN CYTOMEGALOVIRUS INFECTION AND THE RISK OF CARDIOVASCULAR DISEASE

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“Once you have made the decision you will not fail, the heart and body will follow.”

Kara Gaucher

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ABBREVIATIONS

AHA – American Heart Association

BSA – bovine serum albumin solution

CVD – cardiovascular disease

DAPI - 4',6-diamidino-2-phenylindole

DNA – deoxyribonucleic acid

EC – endothelial cell

eNOS - endothelial nitric oxide synthase

ERGIC – endoplasmic reticulum Golgi intermediate compartment

HBV – hepatitis B virus

HCMV – human cytomegalovirus

HCV – hepatitis C virus

HES - haematoxylin-eosin-saffron staining

HIV – human immunodeficiency virus

HKG – house keeping genes

HLA - human leukocyte antigen

HSP – heat shock proteins

ICAM - intercellular adhesion molecule-1

IE – immediate early proteins

IFN – interferon

IRL – internal repetitive region long

IRS – internal repetitive region short

LDL – low-density lipoprotein

LILRB1 - Leukocyte Immunoglobulin-Like Receptor B1

MCP – macrophage chemoattractant protein

MCSF - macrophage colony-stimulating factor

MHC – major histocompatibility complex

MICA/B - MHC class I chain related-proteins A

MIE – major immediate early

MIEP – major immediate early promoter

NEC – nuclear egress complex

NK – natural killer cells

NKG2D – natural killer cell activating receptors like

NO – nitric oxide

PBS – phosphate buffered saline solution

PCR – polymerase chain reaction

PE – preeclampsia

RNA – rybonucleic acid

ROS – reactive oxygen species

SMC – smooth muscle cell

TNF – tumor necrosis factor

TRL – terminal regions long

TRS – terminal regions short

ULBP – unique long binding protein

VCAM – vascular cell adhesion molecule

VPK – viral protein kinase

WHO – World Health Organization

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CHAPTER 1. INTRODUCTION

1.1 The global impact of cardiovascular disease

Cardiovascular disease (CVD) is the main cause of death in adults worldwide according to WHO (World Health Organization) [31]. The group of cardiovascular diseases includes almost 13 different entities. In 2019, 85% of the 17.9 million deaths due to CVD worldwide were related to heart attack or stroke[31]. People's lifestyles and socio-economical development significantly impact the number of CVD cases and CVD-related deaths. Thus, low-income and middle-income countries register more than 75% of all deaths from cardiovascular causes [31,49,173]. The economic burden of CVD in the European Union was estimated to be over 200 billion euros annually in 2021 [49]. Due to its importance as a health and economic problem, CVD is approached with great care nowadays. The main targets of all programs designed to reduce mortality from cardiovascular disease are lifestyle changes and reduction of cardiovascular risk factors, as well as better access to medical prophylaxis.

1.2 Atherosclerosis - the underlying mechanism of cardiovascular disease

The term atheroma comes from the ancient Greek language (g. athíroma) and means porridge[2]. The doctor Albrecht von Haller introduced it in the specialized literature in 1755 to describe the degenerative process present within the arterial wall [42,53]. From the 18th century until the middle of the 20th century, the notion of atheroma received several interpretations from the clinicians and anatomopathologists of the time, these being divided between the inflammatory nature of the lesion and the simple proliferation of smooth muscle cells within the intimal layer of the arterial wall[2]. The theory of the involvement of lipids in the development of atheroma plaque, which still stands today, was issued in 1913 following experiments on laboratory animals, by Anitschkov and Chalatov[89,152]. The next step regarding this theory was only achieved in 1970 by Brown and Goldstein who highlighted the fact that the receptor for LDL initially discovered by them is not involved in the transformation of macrophages into foam cells[64]. More than10 years later, Daniel Steinberg revolutionize the view on atherosclerosis by identifying the extremely important role played by the oxidized fragments of LDL cholesterol in the generation of atheroma plaque[148]. Meanwhile, in 1976, Russel Ross defines the first stage in the appearance of atherosclerosis as "discrete endothelial injury"[137], bringing atherosclerosis as a chronic inflammatory disease back into the spotlight. The '80s represent the crossroads regarding studies related to atherosclerosis, thus giving birth to

two research directions, one that targets active lipid mediators (ie LDL) and the second that refers to other factors that can cause inflammation at the level of the arterial wall (different infectious agents, autoimmune diseases). In 1995, the American Society for the Study of the Heart (AHA - American Heart Association) publishes the first classification of atherosclerotic lesions that divides them into six groups depending on the histopathological aspect[147]. The intense study of the process underlying the diseases with the highest degree of death in the world, leads to the elaboration by the AHA of a new classification of atherosclerotic lesions in the year 2000 (Figure 1) [174].

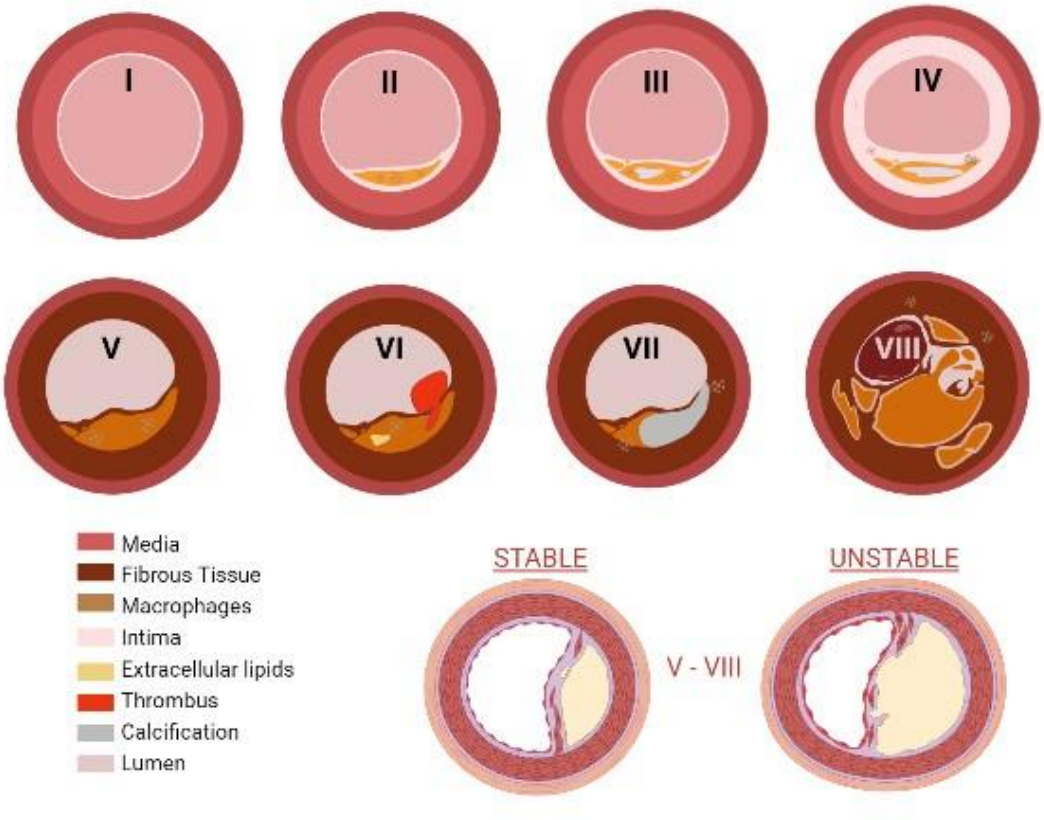

Figure 1. Atherosclerotic lesions classification according to AHA [174]

I Initial lesion that may seem normal to histology evaluation but with isolated foamy cells presence; II fatty traces with multiple macrophage foamy cells; III intermediate lesion with intracellular lipid accumulation as well as isolated extracellular lipid pools; IV mature atheroma plaque with extracellular lipid core; V mature plaque with more than one lipid core covered by a fibrous cap; VI complicated plaque with fibrous cap rupture and possible thrombus; VII calcified plaque; VIII extend fibrosis with little or absent lipid deposits and important luminal narrowing. UNSTABLE plaque is characterized by an important necrotic core and a fine fibrous capsule that can break easily; stage V to VIII plaques have the potential to become unstable.

Nowadays, atherosclerosis is defined as a progressive chronic inflammatory process within the arterial wall of middle-size or large arteries that may start in utero[105,109]. It is characterized by vascular remodeling with thickening and stiffening of the arterial walls. This complex process is due to the accumulation of lipids and secondary fibrosis at the level of the arterial wall, because of the innate and adaptive immune response[103,135,152]. Depending on the age at which the initial lesions appear and the type of immune response, the process of plaque formation may extend through one or more decades or it can be very fast, within a few months.

1.3 Pathogenesis of atherosclerosis

The wall of a normal artery is made of three layers. Thus, from inside to outside we find tunica intima, tunica media, and adventitia, figure 2[133].

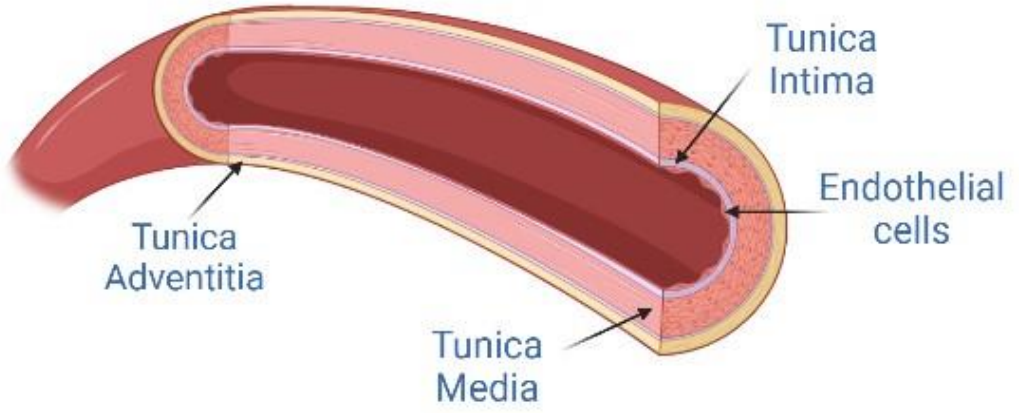

Figure 2. The arterial wall layers – created with BioRender.com

The internal layer of the arterial wall, called endothelium acts like an insulating layer of the blood stream. It is made of vascular endothelial cells and it plays a vital role for arterial homeostasis. The middle layer of the arterial wall consists of smooth muscle cells. This is an elastic structure with a key role in arterial wall adaptation to blood flow pressure variations. Adventitia, the outer layer, is made of collagen and its role is a supportive one[175].

The key mechanism in atherosclerosis is endothelial impairment as an initial point in plaque development [24,78]. Studies carried out in the last two decades have identified the following main triggers for endothelial lesions[78,152]:

a. ox LDL accumulation
b. low production of nitric oxide
c. mechanical factors
d. infectious agents
e. heat shock proteins

During plaque formation, a few different stages were described: lesion, the release of proinflammatory factors (cytokines, adhesion molecules, growth factors), platelet aggregation, leukocytes, macrophages, dendritic cells, and lymphocyte T migration at the level of vascular intima with its thickening. The continuous cell influx, followed by a consistent inflammatory response leads to plaque maturation. At this stage, the plaque is composed of a soft, necrotic core, surrounded by the cells of the immune system[24]. As a physiologic phenomenon limiting this inflammatory process, the mature plaque will be covered by smooth muscle cells migrated from the vascular media and a collagen-rich matrix, resulting in a mature plaque with a soft core and a fibrous cap. The continuous presence of proinflammatory factors can cause plaque lysis with secondary thrombosis and severe clinical complications. Usually, in atherosclerosis, there is a balance between the Th1 cellular immune response and the Th2 humoral immune response, such as any factors interfering with this balance can determine a rapid progression in plaque maturation and plaque instability resulting in the early onset of symptomatology[163].

1.3.1 Factors involved in initiating an atherosclerotic lesion

1.3.1.1 Hypercholesterolemia

The studies conducted so far reveal the essential role of LDL cholesterol molecules, especially ox LDL cholesterol and other metabolic active lipids in association with macrophage cells presence in initial atherosclerotic lesions for both animal and human models[51]. A plaque sometimes may develop in the absence of risk factors like hypertension, diabetes, obesity, or smoking, but never without cholesterol. This lipid molecule is an ubiquitous component of the human body, located within cellular membranes, hormones, and myelin, basically an indispensable element for human body homeostasis[167]. Therefore, it is difficult to establish an exact reference range for either prevention or treatment of atherosclerosis. Over time, based on clinical results, AHA and

ESC developed guidelines for LDL cholesterol targets in healthy and cardiovascular patients decreasing the reference range from one guideline to another. The recommendations of the current ESC guidelines for dyslipidaemias target an LDL cholesterol limit of 100 mg/dl in healthy individuals and a reference range of 55 to 75 mg/dl for patients with different risk factors or already known CVD[176]. Extremely important evidence of LDL cholesterol in plaque formation is represented by the studies conducted on fetal aortas from different aged, aborted fetuses from dyslipidemic mothers compared to those from normolipidemic mothers. Although during pregnancy, the increased levels of cholesterol are physiological due to a high influx of hormones and a rapid rate of cellular division, a value of total cholesterol up to more than 50% from the value before pregnancy, or superior to 300 mg/dl was associated with prematurity, preeclampsia, spontaneous abortion or incipient atheroma lesions of the fetal aorta[117]. Unfortunately, there is no consensus regarding cholesterol levels in pregnancy, and no screening in this direction is made during pregnancy follow-up.

The oxLDL cholesterol molecules determine arterial wall inflammation by activating specific signal pathways and by stimulating chemokine expression like M-CSF (macrophage colony-stimulating factor) and MCP-1 (macrophage chemoattractant protein-1), vascular cells adhesion molecule-1, VCAM-1, adhesion molecules ICAM-1 (intercellular adhesion molecule-1), and selectine E and P, resulting in monocytes and leucocytes recruitment that will infiltrate the vascular subendothelial tissue. From this point, the macrophages will transform into foamy cells that in return will secrete mmacrophages'chemoattractants factors and cytokines responsible for chronic inflammatory response and in the end endothelial cells' destruction with plaque formation[132,161]. It is essential to understand the importance of cytokines in initiating and maintaining an inflammatory response as an indispensable factor for plaque formation and maintenance of plaque stability.

1.3.1.2 Low production of nitric oxide (NO)

The arterial endothelium produces NO from L-arginine under the catalytic action of endothelial NO synthase (eNOS). The most important role of NO within the arterial wall is to act as a relaxing factor within the muscular arterial layer. Nitric oxide synthesis is stimulated by mechanical, pressure factors, or chemical mediators like acetylcholine or bradykinin[78,83]. To date, NO is considered a protective factor against atherosclerosis not only for its direct arterial action but also for its metabolic effects such as reduced

triglyceride circulating levels and oxidative stress, increased mitochondrial activity, and glucose clearance [78]. The increased oxidative stress generated by established cardiovascular risk factors like smoking, dyslipidemia, obesity, hypertension, and diabetes, considerably reduce the bioavailability of NO[36,78,94]. Thus, initial endothelial lesions will appear triggering an inflammatory cascade and the atherogenic process.

1.3.1.3 Mechanical factors

Arterial hypertension represents the main mechanical factor involved both in the initiation and development of atherosclerotic lesions[61]. In certain conditions and between certain values variations in blood pressure are physiologic. Sudden blood pressure variations as well as constantly increased values, especially in elder population can cause a turbulent blood flow and an abnormal arterial wall "stretch" with the occurrence of mechanical injuries in the intima and the induction of an inflammatory response that may underlie the development of the atheroma plaque. The normal blood pressure value is maintained due to the structural integrity of the arterial wall and to the same extent as the renin-angiotensin-aldosterone axis [150]. Increased angiotensin II circulating levels in hypertensive patients will determine an increased release of reactive oxygen species (ROS) and reduced NO synthesis. This context will lead to cellular death, subsequent endothelial injury, and, finally, inflammation [138,145]. In addition, increased circulating levels of angiotensin II promotes overexpression of adhesion molecules, growth factors and proinflammatory cytokines with vascular remodeling as end-point [121].

1.3.1.4 Infectious agents

Due to large evidence regarding their ability to generate an immune response, starting with the 70s-80s, a series of infectious agents were studied in relation to the atherosclerotic process. This included bacteria or viruses able to generate a short-term but aggressive immune response, but especially those agents capable to remain latent for undefined periods of time in different cell types and to reactivate, thus generating a less aggressive but chronic immune response[30]. Infectious agents can cause an endothelial injury directly by infecting the endothelial cells (herpes simplex, cytomegalovirus, HIV, adenoviruses) or indirectly by endotoxins or inflammatory chemokines[85].

1.3.1.5 Heat shock proteins (HSP)

HSP also known as stress proteins are a family of proteins that protect cells against apoptosis. These proteins are classified according to their molecular weight, and they are

present in all animal cells, including humans [160]. There is evidence sustaining that these proteins can recognize and bind different antigens in order to transfer them to major histocompatibility complex I or II [115]. In addition, this family of proteins can stimulate specialized cells of the immune system like macrophages and dendritic cells, thus generating a cascade of reactions leading to an immune response. HSP 60 and 70 seem to be expressed by macrophages in early stages of plaque formation; HSP 90 is related to vascular smooth muscle migration and proliferation, while HSP 27 an estrogen receptor associated protein might offer protection by conferring plaque stability. Thus HSP 60, 70 and 90 were mentioned as atherosclerotic promoters, while HSP 27 is rather an anti-atherosclerotic factor[87,152]. In response to the stress generated by dedicated cardiovascular risk factors (smoking, obesity, diabetes, etc.) the endothelial cells express HSP in mitochondria, cytoplasm and in the end on the cellular surface, resulting in a "danger" signal leading to both humoral and cellular immune response [10,129,159]. Considering the presence of HSP in many cellular types and so, a pre-existent immunity to HSP, especially HSP 60, cascades of cross reactivity with a strong autoimmune response will be generated leading to endothelial cells injury as a basic lesion in atherosclerosis[70,159].

1.3.2 Pro and anti-inflammatory cytokines in atherosclerosis - *the biologic role of cytokines*

Cytokines are a large group of glycoproteins expressed by a variety of activated cells. For their mechanism of action cytokines can be compared to hormones, having an autocrine, juxtacrine and paracrine action [14]. The action of cytokines on various cell types depends on the presence of specific receptors on the target cell surface. The different cells involved in the initiation and the development of the atheromatous process express cytokines and may also respond to cytokines' action[152]. Studies conducted in atherosclerosis until now suggest the next role of cytokines:

- alteration of endothelial cells permeability with intercellular junction injury[7,103]
- activation and over expression of adhesion molecules like selectins, VCAM-1, ICAM-1 and ICAM-2 on the surface of endothelial cells [103,112,144]
- release of chemoattractant molecules [135]
- modulation of scavenger receptor expression [19]
- lipid metabolism adjustment [55,103]
- modulation of smooth muscle cells proliferation [8,90]

- maintenance of humoral/cellular immune response balance [60,72]
- cellular oxidative stress stimulation resulting in oxLDL cholesterol [101,166]
- modulation of the expression of extracellular matrix [135,152]
- modulation of neoangiogenesis inside the vascular wall and inside the atheroma plaque[80,113]
- regulation of procoagulant activity and fibrinolysis [24,29]
- regulation of apoptotic process [16]

Table1 shows the main cytokines, chemokines, chemokines receptors and growth factors identified so far in human atheroma plaques, in relation to cells that produce them and according to their pro or anti-atherogenic effect.

Table 1. Cytokines expressed in human atherosclerotic plaques [152]

CELL TYPE / CYTOKINES	Macrophages	SMCs	ECs	Th0	Th1	Th2	Treg	NKT
PRO atherogenic cytokines	TNF α IL-1 IL-12 IL-15 IL-18 IL-32 IL-6 IL-8	TNF α IL-1 IFN γ IL-6 IL-8	IL-1 IL-18BP IL-6 IL-8	IL-2 IL-3	IL-17 IFN γ IL-2	IL-10 IL-13 IL-4 IL-5 IL-6		IFN γ
ANTI atherogenic cytokines	IL-10 TGF β IL-1ra IL-18BP						IL-10 TGF β	
CHEMOKINES/ CHEMOKINES RECEPTORS	MCP-1 /CCL2 MCP-4/CCL13 IL-8/CXCL-8 GRO-α/CXCL1 I-TAC/CXCL11 SR-PSOX/CXCL16	MCP1/CCL2 Eotaxina/CCL11 Mig/CXCL9 SDF1/CXCL12 SR-PSOX/CXCL16 CCR1 CCR2	I309/CCL1 MCP1/CCL2 MCP4/CCL13 Mig/CXCL9 I-TAC/CXCL11 MIF VCAM-1 ICAM-1 E-selectin P-selectin	CCR5	CXCR3	CCR4	CCR4 CCR8	
GROWTH FACTORS			SCF IL-3 GM-CSF G-CSF M-CSF					

*SMCs-smooth muscle cells ; ECs – endothelial cells ; ACs – adipose cells ; Th – T helper lymphocytes ; Treg –regulatory T cells ; NKT – natural killer cells.

1.4 Preeclampsia and cardiovascular disease

Preeclampsia (PE), a hypertensive pregnancy disorder, is a leading cause of both maternal and neo-natal mortality and morbidity worldwide[139], with a remarkable economic burden on healthcare systems[59,73]. Complicating about 5% of all global pregnancies[1,13] preeclampsia is characterised by the onset of pregnancy hypertension with proteinuria, occuring after 20 weeks of gestation, that sometimes may lead to HELLP syndrome (hemolysis, elevated liver enzymes, low platelet count) or eclampsia (severe pregnancy hypertension with brain oedema and seizures) [177]. Preeclampsia has been often associated to cardiovascular complications later in life [4,12,136]. A global risk of stroke estimated to 14.5 times higher has been described in patients with a history of preeclampsia compared to controls [4]. Both preeclampsia and cardiovascular disease share a complex vascular impairment and remodeling. The full mechanism of preeclampsia is still unknown, but there is enough evidence for genetically related risk factors, some of them also important for cardiovascular disease development [71,108], as well as already well-known cardiovascular risk factors like preexistent hypertension, diabetes mellitus, hypercholesterolemia or obesity [153]. Endothelial injury and arterial wall thickening are the main mechanisms of vascular remodeling found in preeclapmsia as well as in cardiovascular disease. In preeclampsia, the trophoblast progenitor cells injury with secondary impaired invasion of the spiral arteries will lead to an increase in vascular resistance with increased blood pressure values of the mother. Inadequate uterine and placental vascular remodeling as well as vascular resistance and shear stress will generate endothelial injuries triggering an inflammatory cascade and initiating the atherogenic process within the spiral arteries[97].

CHAPTER 2. HUMAN CYTOMEGALOVIRUS INFECTION

2.1 Human cytomegalovirus (HCMV) structure and genome

HCMV is an enveloped, double stranded DNA virus, the largest member of the *Herperviridae* family, isolated for the first time in 1956. Together with the roseola virus (HHV6) and HHV 7, it is part of the *betaherpesvirinae* subfamily.

2.1.1 Viral envelope

The viral envelope is a lipid bilayer derived from the membrane of the target cells. Different types of glycoproteins are found on the surface of the viral envelope, grouped in three conserved glycoprotein complexes: gcI, gc II and gc III [67]. gB belongs to gcI group. It is encoded by UL 55 gene and it is a fusion protein with an important role in attaching the virus to the target cell membrane and internalization. gM and gN proteins belong to gcII group. They are encoded by UL 100 and UL73 genes. They have no role in the penetration of the virus into the cell, but they are key factors for viral maturation. gN is an extremely variable protein with one particular strain, gN4, presenting an increased risk for the development of congenital infection. gH, gL and gO proteins belong to gc III group. They are encoded by UL 75, UL 115 and UL74 genes. Together with gB, gH and gL participate in the attachment of the virus to the host cell membrane, fusion and internalization. gO it is also involved in virus internalization within the host cell. Due to the previously described functions, all these envelope proteins represent the main targets of neutralizing antibodies [118,178].

2.1.2 Viral tegument

The viral tegument is an amorphous protein structure located between the capsid and the viral envelope. Many viral proteins have been described within the viral tegument structure. The role of these proteins is partially known until now[81]. However, the major protein found within the tegument is pp 65 (UL83). This protein is not mandatory for viral replication but it appears to have a major role in the evasion of innate and adaptative immune response[154]. Due to the large amount in which it is found in the tegument pp65 it is also used as a marker of identification for CMV infection. Other tegument proteins like pp71, pp150 or pp28 have been described as having important roles in gene expression, viral assembly and release [154].

2.1.3 Viral capsid

The viral capsid has an icosahedral shape with 162 capsomere. It is made up of 60 asymmetric units. Each asymmetric unit includes 16 major capsid proteins (MCP), pUL86, and 16 small capsid proteins (SPC), pUL 48/49 [157,168]. The icosahedron's walls are composed of 150 hexons and 12 pentons creating a structure with a thickness of almost 15 nm. The capsid assembly process gives rise to three different forms of capsids A, B and C. Only C form capsids contain HCMV-DNA. A and B forms are impaired capsids with no genetical content [178].

2.1.4 Viral genome

HCMV has a large, liniar, double stranded DNA genome of 236 kbp [45]. Viral DNA is made of two unique regions UL – unique long – and US – unique short. In addition, within the genome there are also terminal regions ,TRL, for UL and TRS, for US as well as internal repetitive regions IRL for UL, respectively IRS for US. The schematic description of the organization of the viral genome is *ab-UL-b'a'c'-US-ca* (figure 3).

Figure 3. Schematic representation of the HCMV genome

a – TR (terminal region long and short respectively); b, c, a', b', c' IR (internal repetitive region long and short respectively); pac 1 and pac 2 – cis acting signal structures responsible for DNA cleavage and packiging initiation

The *a* regions are TRL and TRS sequences respectively that contain two conserved cis-acting signal areas *pac1* and *pac2*. These last two structures are recognized during viral replication and have a role in the cleavage and packaging of the genetic material. *b* and *b'* sequences do not have a specific role in the course of viral replication and are rather the result of the extensive study of the virus in cell cultures. *c* and *c'* are HCMV conserved areas which contain a set of IE genes [178].

2.1.5 The HCMV life cycle

HCMV can infect a wide variety of cell. Depending on the type of infected cell, the duration of a complete replicative cycle is between 48 and 72 hours. The first stage of viral

replication is the eclipse phase, which includes adsorption, internalization and decapsidation of the virus within the host cells. The next stage represented by the growth phase consists of the synthesis of immediate early viral proteins, the synthesis of early viral proteins and of viral nucleic acid and the synthesis of late viral proteins. The last stage of viral replication consists in the assembly and release of progeny virions. However, until this moment the complete mechanism of viral replication of CMV remains unclear.

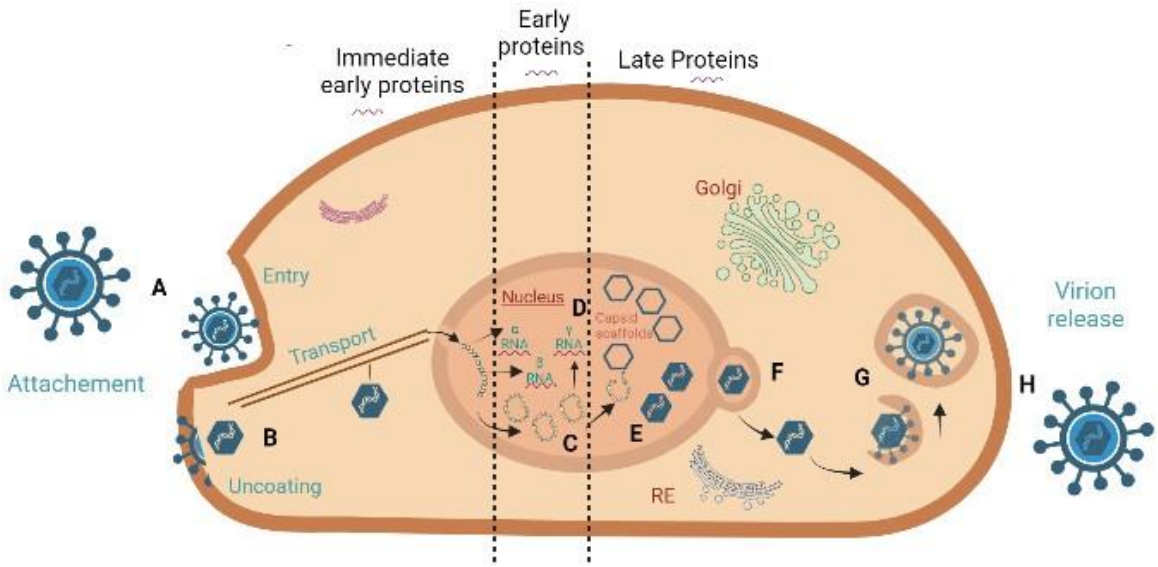

Figure 4. Cytomegalovirus life cycle (created with BioRender.com; adapted after Gatherer et all., 2021[62]). **A** Attachment of HCMV viral particle to the membrane of the host cell through glycoproteins gB and gM and internalization regulated by gH/gL/gO and gH/gL/pUL128/pUL130/pUL131A complexes. **B** Transport to the level of the nucleus through a system of cytoplasmic microtubules. **C** Release of the viral genome inside the nucleus and replication according to the „rolling circle" model. **D** The expression of IE, E and L genes with a role in the identification of the oriLyt domain, initiation of viral replication, generation of new capsids and tegument proteins as well as the assembly and release of progeny virions. **E** Viral encapsidation performed by pUL56/pUL89/pUL51 terminase complex. **F** Translocation of the nucleocapsid through the nuclear membrane by NEC (nuclear egress complex). **G** Maturation and envelopment of virions at the level of the endoplasmic reticulum and Golgi apparatus in the cytoplasmic assembly compartment. **H** Exocytosis release of progeny virions, regulated by UL103.

2.1.5.1 Adsorption, internalization and uncoating

Adsorption is a passive phenomenon by which the viral particles are carried towards the target cells. Enveloped viruses such as HCMV present on their surfaces a series of epitopes (glycoproteins) that allow them to attach from a distance to specific receptors, through a "bridged" model [18]. The first contact between HCMV and the cell is made via envelope glycoproteins gB and gM that bind heparan sulfate. There is no need for specific recognition receptors in order to complete the attachment to the host cell [76]. gB trimers and gH/gL heterodimers will work together to create a fusion body between the viral membrane and the target cell membrane, or to facilitate endocytosis, so that the virus can enter the cell [156]. For entering epithelial or endothelial cells, HCMV requires the presence of specific envelope glycoprotein complexes like gH/gL/gO and gH/gL/pUL128/pUL130/pUL131A (figure 4A) [123]. Once inside the cell, the virus is carried by intracytoplasmic microtubules to the nucleus (figure 4B). This translocation process via cellular cytoplasm followed by uncoating is still unclear, but tegument proteins like UL47 and UL48 might have an important role [27].

2.1.5.2 Growth phase

2.1.5.2.1 Immediate early genes expression and function

The first step of this phase starts with the expression of immediate early genes (IE), right after the viral genetic material reaches the nucleus of the host cell. Naturally, after the host cell is infected, epigenetic repression will occur. In order for the expression of viral genes and transcription to be possible, it is necessary to suppress the cellular defense mechanisms. Thus, before the viral replication begins, some viral tegument proteins, such as pp71 and ppUL69 will act to supress the repressive action of cellular demethylases, histone deacetylases (HDAC) and methylases system[178]. After this step, the transcription of viral proteins it is possible under the action of RNA polymerase II. The major immediat early (MIE) or IE1/IE2 is the first gene transcribed from a specific promoter (MIEP – major immediate early promoter). Thereby, HCMV viral IE1-p72 and IE2-p86 proteins are the first to be produced during viral replication. Their presence is imperative for the continuation of the viral replication process, as they act together with other proteins and transcriptional factors to suppress cellular defense systems, ensures the regulation of IE genes expression, they establish the sites where the viral DNA synthesis can begin and control the transition from the latency to viral reactivation [5,17,21]. Apart from IE1/IE2, there are 4 other loci within HCMV genome that encode for IE ancillary

proteins. Thus, UL36/UL37, TRS1 and IRS1have an auxiliary but important modulatory role in viral replication, while US3 has a role in viral immunological escape by acting on the major histocompatibility complex type I [41].

2.1.5.2.2 Early genes expression and function

The expression of early genes (E) always occurs after the synthesis and under the control of the IE genes. E genes seem to have a double role in viral replication. On one hand, they are important for the viral DNA synthesis and, on the other hand, they are blocking the host cell's life cycle. The most studied loci of the HCMV genome that encode for E genes are UL112/UL 113, UL54 and UL4. Corroborated with the action of proteins synthesized by IE genes (TRS1/IRS1 and MIE), UL112-UL113 transcription factors (pp34, pp43, pp50, pp84) act as transactivators of UL54 promoter [86]. UL54, the pol gene is responsible for the expression of DNA polymerase without which viral replication cannot take place [25,39] (figure 4D). UL4 is an ancillary protein that encodes for gp48, a protein whose role is not very well known . It might inhibit the translational process by a short upstream open reading frame (ORF) [47,54].

2.1.5.2.3 HCMV DNA synthesis

HCMV DNA synthesis takes place inside the nucleus of the host cell according to the „rolling circle" model (figure 4C), after 24 hours from infection. The origin of viral replication is in the oriLyt complex region located between UL57 and UL69 genes. The identification of the oriLyt region is made by UL84, an ancillary IE gene that binds to IE2 gene. ppUL84/IE2-p86 complex identifies the oriLyt domain in order to initiate viral replication[165,178]. To ensure an optimal viral replication process, the presence of a set of specific conserved genes is necessary: UL105, UL72, UL102, UL54, UL44, UL57, UL84, IE2, TRS1/IRS1 and UL112-UL113 [6,35,104]. After oriLyt region identification, the HCMV dsDNA strand separation occurs under the action of the helicase-primase complex (UL105, UL70 and UL102). Subsequently, new copies of viral DNA will be generated by UL54 polymerase. The latter's activity is stimulated by its accesory protein UL44 and by the activation of MIE, TRS1/IRS1 and UL112-UL113 genes [6,104]. After polymerization ends, a terminase complex of the HCMV DNA concatemer will cleave the newly synthetized genetic material into unit-length genomes and then pack it. Hetero-oligomer pUL56/pUL89/pUL51 terminase complex recognizes the packing signals or *pac* sequences on concatemeric viral DNA and makes a first cut, generating a free end. It then recrutes empty capsides and start the viral DNA translocation (figure 4E). The terminase

complex will make its second cut after a unit-length genome is completely traslocated to the capsid. At this moment pUL56/pUL89/pUL51 will dissociate and will detach from the progeny virion [26,96].

2.1.5.2.4 Late gene expression and function

There are early late (EL) and true late (L) genes expressesed within HCMV genome after viral replication begins. The difference between them consists mainly in the fact that EL can be expressed even in the presence of inhibitory factors for DNA synthesis. Among these genes UL99 encodes for tegument protein pp28, UL75 encodes for envelope protein gH. The transcription products of the late genes are basicaly involved in capsid maturation, DNA encapsidation, maturation and release of progeny virions from the host cell[178].

2.1.5.3 Maturation and release of progeny virions

The process of virus maturation starts right after the newly synthetized HCMV DNA is translocated within the capsid. There are 3 different kind of capsids, as already mentioned before, A that lack both scaffold and genetic material, B that only lack genetic material and C, the only viable capsid that contains HCMV DNA and that will continue the maturation process. Capsids full of viral genetic material will receive some major tegument proteins, pp71 and pp65, within the nucleus [3]. Furthermore, a HCMV nuclear egress complex (NEC) will carry out the capsids translocation from the nucleus to the cytoplasm (figure 4F) [99]. This process of passing through the nuclear membrane is accompanied by the attachment of ppUL53 proteins, the component of NEC, to the viral capsid and by the lamina propria proteins phosphorylation by the viral ubiquitous protein kinase (VPK) UL97. Stabilization of the nucleocapsid in the cytoplasm is carried out by pp150 (UL32) and UL96[151]. UL97, viral protein kinase (VPK) ensures the transport of the nucleocapsid to the endoplasmic reticulum Golgi intermediate compartment (ERGIC), inside the cytoplasmic assembly compartment in order to get an envelope[91,178]. Here, the envelopment is produced under the simultaneous action of the following genes UL99, UL94, gM/UL100:gN/UL73, UL71, UL88, UL47, UL48 and VPK (UL97) (figure 4G) [178]. From this point, the new viral particles will be transported through the vesicles of the Golgi apparatus to the level of the cell membrane. The release of progeny virions and dense bodies will be in the end achieved by exocytosis (figure 4H), regulated by UL103 [3].

2.2 HCMV latency and reactivation

The mechanistic basis of HCMV persistence is poorly understood. However, latency implies the persistence of the virus throughout life. It is an escape mechanism from the surveillance of the immune response. During latency the virus does not replicate. It maintains its circularized genome as a minichromosome associated with cellular histones.

HCMV can infect and establish latency in almost any cell type. Yet, the main latent reservoirs identified so far are CD 34+ myeloid precursors [98,106]. Four genes located in the UL133-UL138 locus have been intensively studied regarding latency and reactivation: UL133, UL135, UL136 and UL138 (figure 5). Of these four, UL138 seems to be the most pro-latency by „silencing" the MIEP and increasing the levels of TNF-α [110]. On the other hand, UL135 transcripts interact with host adaptor proteins and regulate EGFR, thus being a promoter of HCMV reactivation from the latency [110,134].

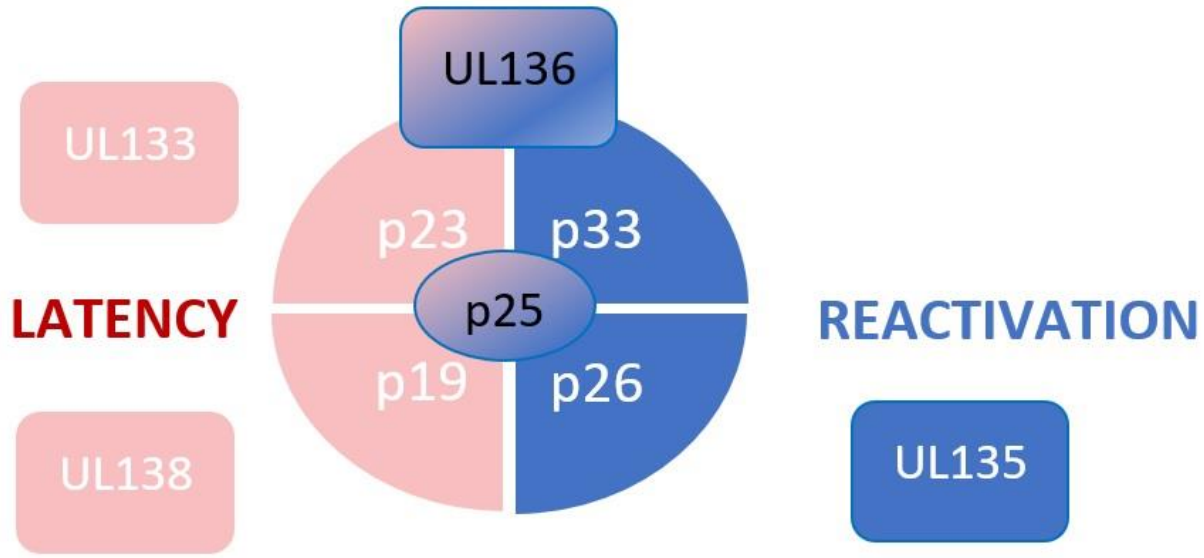

Figure 5. UL133-UL138 genes expression and their role in latency or reactivatation. UL133 and UL138 supress viral replication. UL135 has pro-reactivation properties. UL136 is transcribed into 5 different isoforms and it function as a transitional element between latency and replication – p23 and p19 join the suppressive group of viral replication; p33 and p26 favors viral replication. p25 acts opportunistically and its function is still unclear.

Naturally latent CD34+ cells carry viral genome, but they do not express major IE genes transcripts IE-p86 and IE-p72. MIE genes are expressed only after CD34+ latent cells transform, in special conditions into macrophages or dendritic cells, thus causing viral reactivation [142]. The mechanism of latency/reactivation switch is not fully understood,

but however but in any case, the key is the inhibition of the suppressive action on the MIE. Studies developed in this direction demonstrated that viral reactivation involves a cascade of changes at the level of the host cells. Cellular mediators, especially pro-inflammatory factors can activate MIEP. In fact, any condition causing immune supression can determine MIEP NF-kB mediated activation with subsequent viral reactivation[57,142].

2.3 HCMV immune evasion

Primary HCMV infection activates both innate and adaptative immune system. Innate immunity is mediated mainly through NK cells and toll like receptors (TLR) while the adaptive immune response consists mainly in the activation of CD4+ and CD8+ T lymphocytes. In order to produce latency and reactivation, HCMV has developed complex mechanisms of immune evasion. However, it is very important to emphasize that these mechanisms are still limited and counterbalanced by an immune response from the host, as primary HCMV infection and even latent infection in immunocompetent subjects is usually asymptomatic. The two principles by which HCMV succeeds in deceiving the immune system are the impairement of the host's antigen processing and presentation system and the activation of a set of inhibitory molecules and activators of different kind of receptors at the level of NK cells and lymphocytes T. Thus, the transcription products of the genes located between US2 and US11 interact with the normal expression of MHC I and II. Aditionally, MHC I expression is higly influenced by viral tegument proteins pp71 and pp65. The MHC I and II malfunctioning will lead to the disruption of recognition of infected cells by CD8+ and CD4+ respectively [77,142]. Furthermore, to avoid recognition by NK cell, HCMV UL18 encodes an MHC-I like structure that binds an inhibitory receptor LILRB1 and UL40 transcript enhances HLA-E expression on the surface of the infected cells. In order to reduce NK cells cytotoxicity, HCMV downregulates cellular stress ligands expression such as ULBP, MICA/B, CD112, CD155, CD48, CD58 and sometimes may directly trigger the NK activating receptors like NKG2D [56].

2.4 Epidemiology and pathophysiology of human cytomegalovirus infection

2.4.1 HCMV seroprevalence, transmission and clinical aspects

HCMV is an ubiquitous virus capable to infect a wide variety of cells and to establish lifetime latency with an undetermined number of reactivations. The seroprevalence of this infection is strongly influenced by the socio-economic status, so it varies between 40% in high-income countries and over 90% in low-income countries[172]. The infection can occur at any age and is transmitted through contact with biological fluids, sexual

intercourse, blood transfusions, bone marrow or organ transplantation. In immunocompetent hosts HCMV infection is typically asymptomatic [65]. Post blood or blood products transfusion a mononucleosis-like syndrome may appear. Immunosuppressed patients may develop life-threatening disease during primary infection or reactivations. In HIV/AIDS patients, HCMV infection causes retinitis and encephalopathies. Post bone marrow or solid organ transplantation the clinical picture is more serious, HCMV infection can cause pneumonia with high mortality, hepatitis or colitis [68].Maternal-fetal transmission is the most important from the point of view of clinical consequences. HCMV is an important teratogenic agent included in the TORCH screening protocol of pregnant women in case of viral symdrom or ultrasound abnormalities. It is to date the most common cause of congenital infection [32]. Prevalence of newborn infection is estimated between 0.2% and 6,1% worldwide [52]. HCMV mother-to-child transmission is possible in utero, as HCMV can cross the maternal-fetal barrier and infect the placenta, thus causing haematogenic transplacental congenital infection. It can also be transmitted during labor or by breast-feading, resulting in non-teratogenic infection, usually mild or asymptomatic except in premature infants that can develop sepsis-like syndrome [179]. When a woman acquires a primary HCMV infection during pregnancy the probability of maternal-fetal transmission is estimated to 30 to 40%. Secondary maternal infections (reactivations or reinfections) can transmit, and even if transmission rate is lower they are responsible for at least half of the congenital HCMV cases worldwide [125]. The maternal-fetal infection has an important clinical impact. Considering the link between viral replication and the cellular phase, the moment when the child is infected is very important. Thus, if the primary infection occurs in the first trimester of pregnancy, the virus is teratogenic and induces malformations, often incompatible with life, causing damage to the brain, with severe neurosensorial sequelae (microcephaly, mental retardation, chorioretinitis, blindness and deafness). HCMV is to date, the number one infectious cause of deafness and motor retardation. If the primary infection occurs in the second trimester of pregnancy, it can still produce the disease with cytomegalic inclusions of the newborn, manifested mainly by hepatosplenomegaly, jaundice, hemolytic anemia, thrombocytopenia, purpura, and petechial eruptions. If the primary infection occurs at the end of pregnancy or after birth, the newborn will present reduced symptoms, or the infection may be asymptomatic [28,32]. Of all infected newborns only 10 to 15% will have symptoms at birth. Unfortunately, almost the same

percentage of babies with no symptoms at birth may develop different neurosensorial pathologies later, in the first five years of life [52].

2.4.2 HCMV infection and disease diagnosis

A wide variety of biological specimens like blood, saliva, cerebrospinal fluid, tears, solid biopsies or amniotic liquid can be used for viral detection. A pathognomonic intranuclear "owl eye" inclusion bodies cytopathic effect can be observed when HCMV is isolated in cell cultures [100](figure 6), but also in organ biopsies in case of CMV disease. Immunoperoxydase staining using antibodies against immediate early antigens enhance the sensitivity and specificity of diagnosis. This anatomopathologic examination of cytomegalic inclusion cells is necessary to confirm CMV disease in immunocompromised patients and to differentiate graft damage from CMV disease in transplanted organs. End - organ infection results from CMV dissemination in blood which precedes the disease and progressively increase. Standard IF staining for CMV pp65 antigen can reveal CMV phagocyted by polynuclear cells (antigenemia). CMV genome can also be detected and quantified by Q-PCR from blood or plasma, with standardized results in International Units. This diagnostic tool used to follow-up immunocompromised patients allows preemptive antiviral treatment when viral load increase, to prevent CMV disease. Blood dissemination is present and transitory during primary infection but also during some reinfections or reactivations. In pregnant women it can result in placental infection, localized, or transmitted to the fetus.

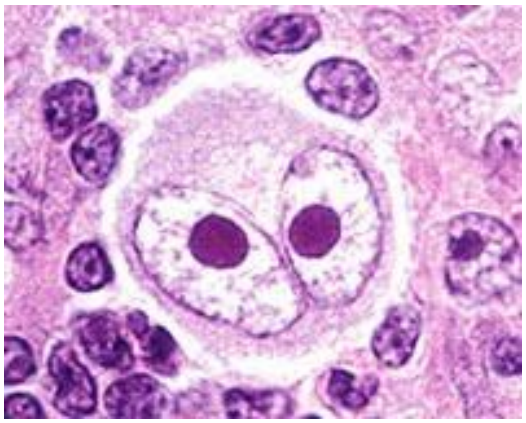

Figure 6. "Owl eye" intranuclear bodies in a human HCMV infected cell. Source :https://www.medicalopedia.org/3153/owls-eye-appearance/

Serology is the first choice when screening pregnant women for HCMV infection. IgG immunity against HCMV normally provides protection for maternal-fetal transmission, while positive IgM antibodies raise the suspicion of an active, potentially transmissible maternal-fetal infection. In this situation, an avidity test is needed to confirm primary

infection, which is associated with the highest risk of maternal-fetal transmission (30-50%). Reinfection and reactivation can also lead to maternal-foetal transmission. Transmission rate does not exceed 18% [116], although, by their frequency in some seropositive populations with high rates of circulating virus, these secondary infections can represent half of the congenital infections [92]. In case of echographic abnormalities, PCR in amniotic fluid is key for congenital infection diagnosis in utero and at birth. At birth, HCMV in newborn urine before 3 weeks of life signs the congenital HCMV infection, thus, eliminating post-partum acquired infection that are non malformative.

2.4.3 Mechanistic basis for placental HCMV infection

The placenta is an organ with a complex structure. Its physiology and physiopathology however are still unclear. Due to the important teratogenic potential HCMV infection is one of the most studied infections in relation to the placenta. Fetal damage in HCMV congenital infection can occur directly through lesions on the fetus, or indirectly, by infecting the placenta with subsequent immune-mediated vascular remodeling and perfusion disorders. Thus, an infected placenta produces intrauterine growth restriction, spontaneous abortion or premature birth rather than the actual transmission of the infection to the fetus [119]. One of the most important roles of the placenta is that of the maternal-fetal barrier or filter. During pregnancy, the maternal blood does not mix with the fetal blood, but only comes in contact through the placenta. For a good functionality of the maternal-fetal interface, a perfect placentation process is necessary, which primarily means the optimal invasion of the uterine wall by the cytotrophoblast and vascular remodeling of the spiral uterine arteries within the first trimester of pregnancy. During the second and the third trimester a complex maternal-fetal vascular network is available and serves for the good development of the fetus [119].

HCMV infects the trophoblast progenitor cells and replicates within cytotrophoblast. Therefore, it impairs cytotrophoblast differentiation and invasion within the arterial wall and spiral arteries, leading to poor placentation and important damage of the maternal-fetal vascular network (figure 7). This mechanism explains the severity of the first term HCMV congenital infection compared to the second and the third trimester of pregnancy when the vascular connections are already generated and functional. The hypoxia and inflammation induced during first term HCMV infection will activate a physiopathological defense mechanism with the increase of chorionic villi number and the increase of placenta's

weight with high oxygen requirement, but without being able to block the proliferation of the infection [126,127].

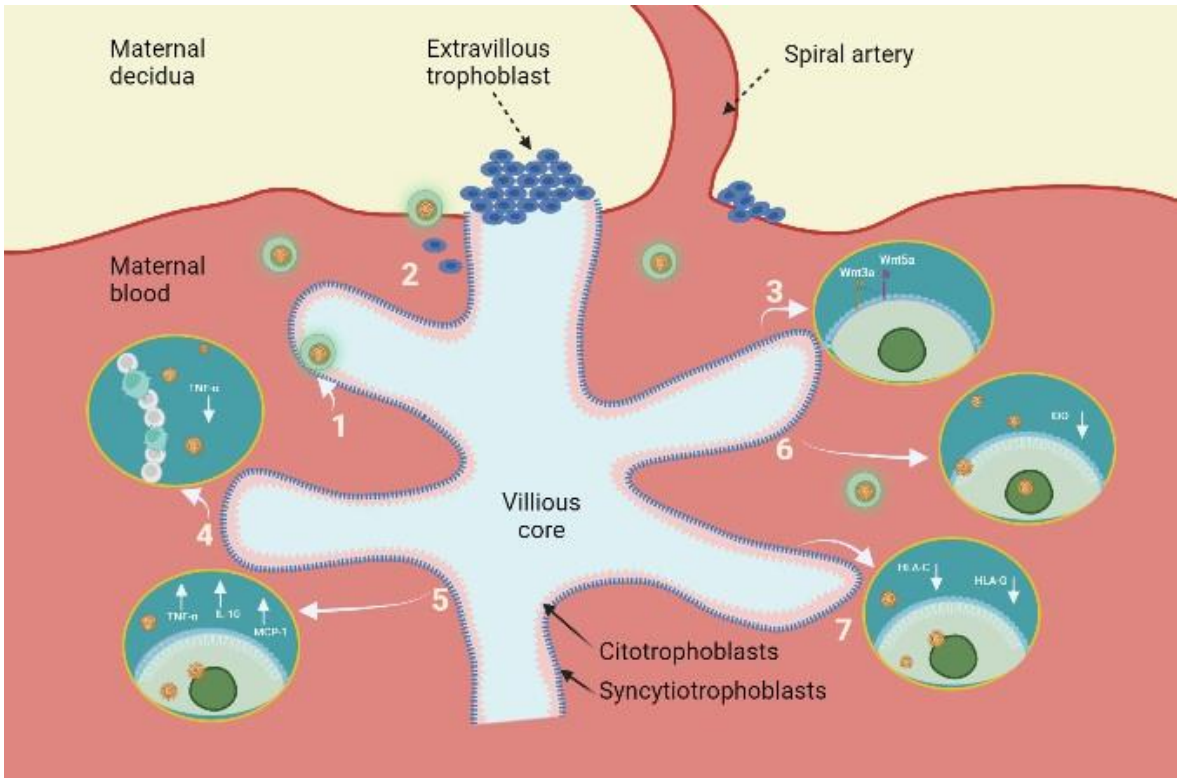

Figure 7. Mechanisms of placenta infection by HCMV (created with BioRender.com, adapted after A. Njue et all.,2020, [119] **).**

1. Infection of trophoblast progenitor cells. 2. Disruption of extravillous trophoblast invasiveness at the level of the uterine wall. 3. Alteration of trophoblast migration by Wingless signaling pathway (Wnt) impairement. 4. TNF-α induced trophoblast apoptosis. 5.Cytokine mediated trophoblast impairement. 6. Dysruption of maternal-fetal tolerance by IDO inhibition. 7. Maternal-fetal tolerance alteration by trofoblast MHC I downregulation. CMV = cytomegalovirus; EVT = extravillous trophoblasts; HLA = human leukocyte antigen; IDO = indoleamine 2,3 dioxygenase; IL-10 = interleukin-10; MCP = monocyte chemoattractant protein; MHC = major histocompatibility complex; ROR2 = tyrosine kinase-like orphan receptor 2; TBPC = trophoblast progenitor stem cells; TCF/LEF = T-cell-specific factor/lymphoid enhancer-binding factor; TNF = tumor necrosis factor; Wnt =Wingless.

HCMV impaires trophoblast differentiation and invasiveness by activating peroxisome proliferator-activated receptor γ, PPARγ, and thus downregulating the expression of integrins α1, β1, matrix metalloproteinase MMP-9 and many other adhesion molecules.

Furthermore, HCMV-IL10 molecule plays an immunosuppressive role and will once again reduce the invasive capacity of the extravillous trophoblast. All these will result in a deficient vascular remodeling with hypoxia and fetal distress [119,127]. In addition, HCMV regulates trophoblast differentiation, migration and invasiveness by inhibiting Wingless signaling pathway (Wnt) [15,82] and by generating a hyperexpressed immune response. HCMV infection increases NK cell cytotoxicity by downregulating MHC I expression on the trophoblast surface. It can also induce apoptosis by stimulating TNF-α expression and disrupt the maternal-fetal immune tolerance by inhibating the activity of indoleamine 2,3-dioxygenase (IDO) [119,126,127].

2.5 Review article

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Review

The Role of CMV Infection in Primary Lesions, Development and Clinical Expression of Atherosclerosis

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Abstract: The number of deaths related to cardiovascular disease is increasing every year, despite all available therapies and the aggressive campaigns for lifestyle modification and prevention of risk factors. Atherosclerosis is a complex process underlying cardiovascular disease. Cytomegalovirus (CMV) is often associated to atherosclerosis and its clinical expression such as coronary heart disease, stroke, or peripheral artery disease. CMV infection may promote acute atherosis within placentas from women with preeclampsia and it may also accelerate atherosclerosis in HIV-infected and organ-transplanted patients. This review focuses on the current scientific evidence for the role of CMV infection in the development of acute atherosis and atherosclerosis from placentation throughout life.

Keywords: cytomegalovirus; atherosclerosis; cardiovascular disease

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1. Introduction

Atherosclerosis is the pathophysiological phenomenon underlying myocardial infarction, stroke, and peripheral artery disease. It is a progressive inflammatory process that can occur in people of all ages [1–3], even in childhood [4]. Atherosclerosis is characterized by the accumulation of lipids and secondary fibrosis of the arterial wall, followed by calcification or plaque instability and eventually plaque rupture with clinical events. Both the innate and adaptive immune responses play an important role in the evolution of the disease [5]. The process of plaque formation may extend through one or more decades or can be very fast, evolving within a few months, depending on the type and onset of the immune response. The mechanism of atherosclerosis is still unclear [3,5], although inflammation plays a crucial role and specific lifestyle-related risk factors have a high impact (smoking, dyslipidemia, diabetes, obesity, level of physical activity, and stress). Various infectious agents have been investigated as potential triggers or cofactors for atherosclerosis [6]. Thus, Helicobacter pylori induces chronic systemic inflammation by molecular mimicry. Periodontal pathogens can cause bacteriemia, leading to direct plaque invasion. SARS-CoV-2 generates a cytokine storm and plaque thrombosis. Human herpes simplex virus produces a proinflammatory state with lipid metabolism impairment. Hepatitis C virus increases the expression of cardiac and inflammatory biomarkers. Streptococcus pneumoniae, Chlamydia pneumoniae, and Mycoplasma pneumoniae may induce persistent pro-inflammatory state and platelet activation with subsequent plaque instability [7]. Among the plethora of bacteria and viruses, CMV was frequently detected in both atherosclerotic plaques and in healthy arteries, and its persistence is related to development overtime of atherosclerosis [8].

CMV is an ubiquitous beta herpesvirus, with a global prevalence in general population estimated at 83% [9] and a regional prevalence correlated with the socio-economic level of

each country. During the last decades, CMV was incriminated in plaque formation and cardiovascular disease [10] due to its capacity for infecting endothelial cells, macrophages, dendritic cells, fibroblasts, and even smooth muscle cells, and its lifetime latency in different cellular types including monocytes and hematopoietic stem cells, with multiple reactivations that can trigger a chronic inflammatory status [10–13].

2. Cytomegalovirus and Cardiovascular Disease

2.1. Evidence of CMV's Role in Plaque Formation and Cardiovascular Disease

2.1.1. Epidemiological Studies

The first observations of a possible correlation between CMV and atherosclerosis were made almost 30 years ago. The presence of anti-CMV IgG antibodies within the sera and the presence of CMV antigens within vascular smooth muscle cells were associated with different stages of plaque formation in patients undergoing vascular surgery at that time [14,15]. Since then, the ongoing research on the implications of CMV infection in cardiovascular disease generated rather variable results, suggesting the importance of the study design and population sampling (Table 1).

Most CMV serology-based studies in patients at risk for cardiovascular diseases have delivered controversial results, except for a certain population group such as patients with diabetes. High titers of anti-CMV IgG antibodies correlate to atherosclerosis in type 2 diabetes mellitus patients, after adjustment of other common cardiovascular risk factors [16]. Patients with diabetes mellitus have an increased risk and an accelerated rate of development of atherosclerosis [17]. There might be a bi-directional link between CMV infection and diabetes, as patients with diabetes mellitus have an impaired antiviral immune response [18] that might promote CMV reactivation, while chronic CMV infection is associated with glucose regulation [19] and new-onset type 2 diabetes mellitus [20]. Nevertheless, all these complex interactions need a better understanding and more research studies in order to clarify the link between CMV infection and diabetes and cardiovascular disease.

CMV direct detection by DNA or antigen identification strongly associates viral infection with atherosclerosis and cardiovascular disease as an end-point. Recent studies showed a higher viral load in STEMI patients compared to controls [21] and a direct relationship between T cells activation and the number of CMV-DNA copies inside the atherosclerotic plaques in patients with peripheral artery disease [22]. A higher number of CMV-DNA copies were detected in patients with preexistent cardiovascular risk factors and were associated to acute coronary syndrome, suggesting that CMV reactivation may lead to the progression of atherosclerotic lesions, for example, transforming stable angina into unstable angina or myocardial infarction [23]. CMV-DNA was found in aortic plaques, but not in normal artery samples obtained from candidates for coronary artery by-pass graft [24]. Immunohistochemical studies demonstrated the presence of CMV pp65 antigen [25] in plaques obtained from patients with atherosclerosis undergoing vascular surgery [26].

Table 1. Associations between CMV, atherosclerosis, and cardiovascular disease.

Authors	Reference	Population	CMV Determinant	Outcome	Association	Study Type
Adam et al.	[14]	157 caucasian male undergoing vascular surgery for atherosclerosis	Serology	PAD	YES	Case-control
Melnick et al.	[15]	132 arterial tissue samples from atheroslerotic plaques of caucasian patients undergoing vascular surgery for atherosclerosis	IF pp65	PAD	YES	Prospective cohort
Jia et al.	[27]	3328 multiethnic patients but predominantly asians with CAD and PAD with or without surgical indication	Serology CMV DNA IF pp65	CAD and PAD	YES	Meta-analysis (control-case or nested control-case)

Table 1. *Cont.*

Authors	Reference	Population	CMV Determinant	Outcome	Association	Study Type
Nikitskaya et al.	[22]	71 ACS, 26 SCAD, 22 atherosclerotic plaques (PAD)	CMV DNA	CAD and PAD	YES	Case-control
Zhang et al.	[16]	222 hospitalized patient with type 2 diabetes mellitus	Serology CMV DNA	T2DM and ultrasound confirmed atherosclerosis	YES for latent infection only	Prospective cohort
Courivaud et al.	[28]	570 renal transplant recipients	Serology CMV DNA	CAD, PAD, stroke	YES	Prospective cohort
Betjes et al.	[29]	408 ESRD patients	Serology	CAD and PAD	YES	Retrospective cohort
Lebedeva et al.	[21]	33 STEMI patients	CMV DNA	IHD (STEMI)	YES	Case-control
Nikitskaya et al.	[23]	97 CAD patiens	CMV DNA	CAD	YES	Case-control
Heybar et al.	[24]	55 CABG—normal and atherosclerotic samples	CMV DNA	CAD—CABG	YES	Case-control
Wang et al.	[25]	15 paraffin-embedded peripheral artery specimens from patients with ATS	IHC	PAD	YES	Prospective cohort
Ibrahim et al.	[26]	48 biopsies from atherosclerotic plaques	CMV DNA	PAD	YES	Case-control
Hamilton et al.	[30]	8531 white ethnic background with no prevalent CVD	Multiplex serology panel	CVD, IHD, stroke	NO	Prospective cohort

CAD—coronary artery disease, CABG—coronary artery by-pass graft, CVD—cardiovascular disease, ESRD—end-stage renal disease, IHD—ischemic heart disease, IHC—immunohistochemistry, PAD—peripheral artery disease, STEMI—ST-elevated myocardial infarction.

Differences in the worldwide seroprevalence of CMV, as well as in the sample type (blood, atheroma plaques, and vascular wall fragments) and detection technique (serology, immunofluorescence, PCR alone or combined) can account for the sometimes controversial data. Overall, direct detection of viral antigens and/or DNA is a better proof of CMV association with atherosclerosis or cardiovascular disease, compared to serology alone.

2.1.2. HIV Associated CMV Infection and Atherosclerosis

Asymptomatic CMV replication can trigger immune system activation and perpetuate an inflammatory environment that favors the early development of atherosclerosis. This is more evident in immunosuppressed individuals, either transplant recipients or people living with HIV (PLWH). Previous studies in HIV-infected individuals have suggested a link between CMV reactivations and an increased intima-media thickness, demonstrated by higher levels of specific anti-CMV antibodies and CMV-specific T-cell responses [31,32]. Probably, the two pathogens act synergistically to promote the complex process of plaque formation [33]. Macrophages and monocytes involved in the initial "traditional" atherosclerotic lesions are reservoirs for both HIV and CMV and the activation of these cells, with foamy cells formation, represent the main mechanism in plaque initiation. Early stages of atherosclerosis have been detected by coronary computed tomography angiography (CCTA) in HIV individuals, who are at high risk of thrombosis and plaques rupture, leading to HIV-associated acute coronary syndrome [34]. Noncalcified, inflammatory plaques that can lead to myocardial infarction and stroke have been also detected in PLWH [35]. Cardiovascular disease remains an important cause of non-AIDS-related morbidity and mortality during HIV infection even under continuous cART treatment [36]. PLWH successfully treated with cART, with low or undetectable HIV replication, still express regulatory viral proteins (Tat and Nef) that may alter monocyte/macrophages cell function [35], possibly triggering subclinical episodes of CMV reactivation. These, in turn, will maintain or amplify the status of chronic inflammation, T cell activation, and immune dysregulation already present in HIV-infected patients, even in those under long-term suppressive cART or in elite controllers. Supporting this hypothesis, a small randomized placebo controlled trial on the effect of valgancyclovir, a potent antiviral drug, showed a significant decrease in

both CMV DNA and the level of CD8 T cell activation in immunosupressed HIV-infected patients under supressive cART [37]. Moreover, chronic CMV infection is characterized by an unusual expansion of specific memory T cells and is a potent trigger for a particular phenotype of CD4 cells with cytotoxic activity that migrate toward the vascular endothelium, playing an important role in the initial vascular lesions and in the progression of atherosclerosis [38]. This process might be accelerated in HIV-infected patients who have a high level of circulating CD8 T cells with increased expression of CX3CR1, the receptor of vascular-endothelium homing chemokine that can be attracted to endothelial cells inducing persistent activation and dysfunction [39].

2.1.3. Cytomegalovirus and Plaque Formation in Pregnancy

Atherosclerosis may start during placentation, increasing the lifelong risk of atherosclerotic disease in women developing preeclampsia, a hypertensive pregnancy disorder [40–42]. CMV is the leading cause of neonatal congenital infections worldwide and is strongly associated with neurological sequelae in newborns and possibly associated with arterial hypertension, a mechanical blood flow condition that damages endothelial cells leading to preatherosclerotic lesions [43]. Based on the above-mentioned observations, we searched for studies that investigated the possible role of CMV in preeclampsia. Several studies looked at the impact of CMV infection on pregnancy complications associated with hypertension, but the results are quite controversial. Interestingly, some studies suggest that the medium-term risk of cardiovascular events in women that develop a hypertensive pregnancy disorder and their offspings is double compared to controls [40,44]. A significant association was found between CMV IgG sero-positivity and innate immune response in early-onset preeclampsia and the presence of HELLP (H: hemolysis, EL: elevated liver enzymes, LP: low platelet count) syndrome [45]. On the contrary, a small case-control study on hypertensive pregnancies, as well as a meta-analysis that included 2734 women with preeclampsia and 3424 healthy controls concluded that there is no significant relation between CMV infection (evaluated by both PCR and serology) and the onset of preeclampsia [46]. Nevertheless, it is interesting that acute atherosis occurs more often in women with preeclampsia [47] compared to normal pregnancies and other pregnancy complications like growth restriction, spontaneous preterm labor, or fetal death.

The hypothesis of a possible impact of CMV in vascular remodeling and acute atherosis lesions with maternal and fetal long-term impact on cardiovascular disease is very interesting and opens a new research direction on non-conventional risk factors for cardiovascular disease in young and very young patients.

3. CMV Mechanisms of Action in Atherosclerosis

Ever since it was used for the first time in 1755, the term atheroma describes a complex process involving both chronic inflammation and lipid accumulation, associated with vascular smooth muscle cells proliferation within the intimal layer of the blood vessel [48]. The potential mechanism by which CMV might promote atherogenesis remains poorly understood. However, CMV has a concerted impact on the main pathways involved in atherogenesis (Figure 1).

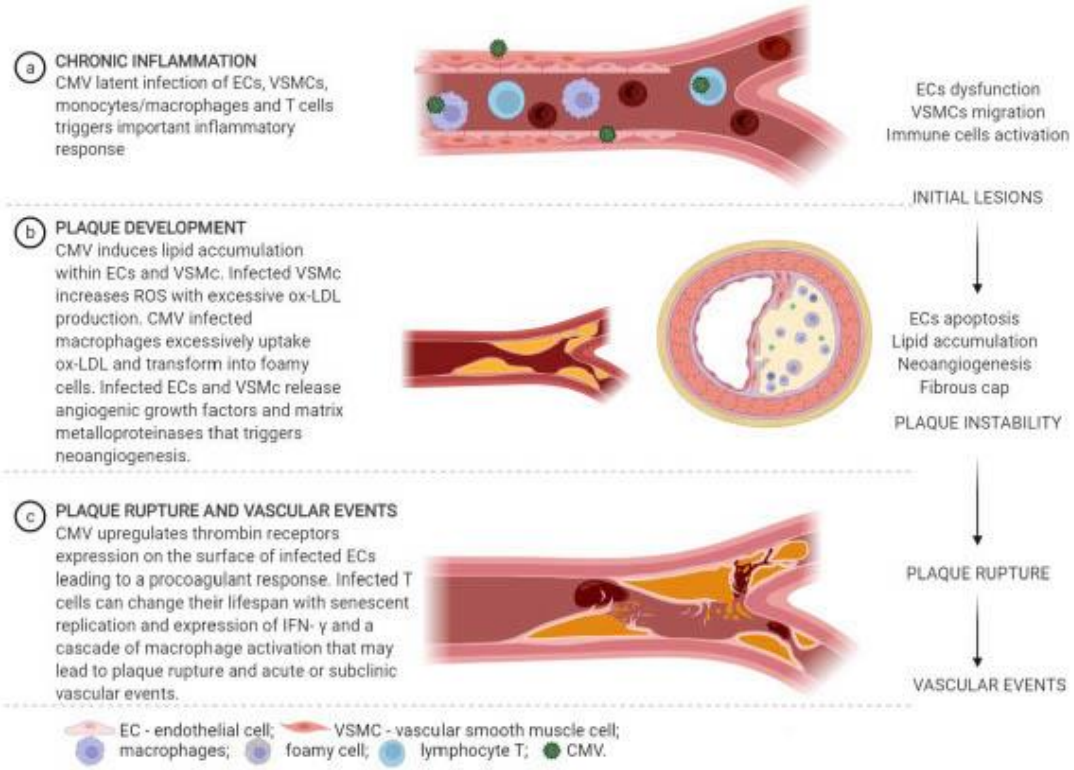

Figure 1. CMV mechanism in atherosclerosis (Created in BioRender.com).

3.1. Oxidative Stress, Lipidogenesis and Endothelial Injury

Oxidized low-density lipoproteins (ox-LDL), which play a crucial role in the early events of atherogenesis, are preferentially recognized by scavenger receptors on macrophages and vascular cells. During CMV infection, the expression of type-B scavenger receptor on the surface of macrophages is upregulated [49]. Reactivation of the latent CMV infection in endothelial cells recruits macrophages and neutrophils by secreting chemoattractant and adhesion factors (MCP-1, VCAM-1, ICAM-1, and CXC), promoting internalization of oxLDL and foamy cells formation. CMV seems to upregulate several important pro-atherogenic molecules responsible for LDL and VLDL cellular uptake and cholesterol synthesis (NPC1L1, HMGCS1, HMGCR, LRP10, 11, 12, and SCARB) and downregulates anti-atherogenic proteins (ApoA1, ApoM, and ApoH) [50]. CMV-infected endothelial cells inhibit angiogenesis and promote abnormal vessel formation, a very important phenomenon already demonstrated in congenital CMV infection [51–53]. The expression of the receptor beta for PDGF (platelet-derived growth factor), an essential factor for the development and the plasticity of the vascular system, is upregulated by HCMV infection, stimulating atherogenesis [54]. CMV UL122 and US28-derived protein are homologous to an 11-aminoacid sequence of HSP60 that seems to produce endothelial cells apoptosis as an early event in atherogenesis [55]. The homology of pUS28 to HSP60 can also determine smooth muscle cells' migration within vascular intima [55]. In vitro, CMV infects vascular smooth muscle cells, resulting in important alterations in the expression of lipid metabolism genes with intracellular cholesterol accumulation [56], possibly by downregulation of SSBP1, which is an important protein involved in wound repair [57].

3.2. Vascular Remodeling

A series of CMV-encoded proteins stimulate inflammation and vascular remodeling (Table 2).

Table 2. CMV encoded proteins and the expression of humoral factors involved in atherosclerosis.

HCMV Protein	Function	Mechanism in Atheromatous Process
UL 7	Early-late gene that is cleaved to release glycosilated ectodomain [58]	Stimulates inflammation in endothelial cells by IL-6 expression and STAT and MAPK pathway activation [58] Increased similarity to the first Ig-like domain of the CEACAM protein family playing a key role in vasculogenesis [58,59]
UL 76	Unclear—highly conserved protein that may cleave nuclear proteins and modulate viral activation or repression [60,61]	Induces IL-8 expression by DNA damage [60]
UL 111A	Encodes human homologous IL-10 cmvIL-10 [62]	Activates CXCL12/CXCR4 and STAT3 signal pathways in monocytes, epithelial cells and fibroblasts [62]
UL 122	IE2 regulatory protein- modulates viral activation and reactivation from latency [55] A protein isoform IE86 encoded by UL122—DNA binding protein that regulates viral gene expression and recruits chromatin modeling enzymes. Essential for viral replication by transactivation of vital viral early promoters probably by direct DNA binding [63]	Homology to heat shock protein 60 (HSP60) and increased monocytes adhesion [55] Supresses the expression of proinflammatory citokines IFN β, RANTES, MIG, MCP2, IE1, GAPDH [64] and may play a protective role in atherosclerosis development
US 28	Expressed on cell surface with high homology to CC chemokine receptor CCR1 facilitating cell-cell fusion [65]	Homology to heat shock protein 60 (HSP60), determines smooth muscle cells migration [55]
UL 128	Envelope protein with immunoregulatory properties, responsible for monocytes and epithelial cells infection [66], thus virus replication and dissemination	Recruits monocytes/macrophages cells by chemoattraction and determines high expression of TNF-α and IL-6 [67]

Antibodies against CMV UL94, a region that encodes the viral tegument protein, are involved in the modulation of thousands of endothelial cells transcripts like adhesion molecules, growth factors, colony-stimulating factors, chemokines involved in leukocytes attraction, angiogenesis, and fibrosis [68]. CMV protein UL7 presents high structural and functional homology to CEACAM1, suggesting a direct involvement of CMV in vasculogenesis [58]. A stable domain in the structure of HCMV UL7 gene is responsible for STAT3/ERK1 MAP signal pathway activation. CMV can bind platelets using TLR2, generating the synthesis of proinflammatory CD40L, IL 1B, and proangiogenic VEGF, and further activating platelets, a cascade that can precipitate atherogenesis [69]. A recent in-vitro study published in 2020 suggests that chronic CMV infection downregulates endothelin 1 (ET-1) expression in endothelial and smooth muscle cells, thus providing additional evidence for the role of this pathogen in vascular impairment [70].

CMV itself can express viral cytokines and chemokines that might play a pivotal role in the angiogenesis and promotion of an inflammatory environment. An extensive homology between CMV and endothelial cell proteins were described and many immune cross-reactions caused by this molecular mimicry were incriminated in endothelial cells apoptosis, in the up-regulated expression of different cytokines and in the pathogeny of vascular damage in autoimmune diseases [68,71].

3.3. miRNA Regulation

During the last decade, small, highly-conserved, noncoding single-stranded RNA fragments called micro RNA (miR) were described as promoters and potential biomarkers of atherosclerotic lesions [72]. miRs were widely investigated and determined to be

regulating different cell functions in atherosclerosis. They play a crucial role in all stages of atherosclerotic process, from the initial endothelial injury and lipid accumulation to angiogenesis, calcification, and thrombosis [72,73]. miR-21, for example, increases NOS phosphorylation and inhibits PPARa and Bcl2, leading to apoptosis and endothelial cells injury [73]. Elevated levels of miR-338-3p were identified in patients with atherosclerosis and were linked to ox-LD-induced apoptosis within endothelial cells [50]. Likewise, miR-126 and miR-127 are related to cell apoptosis by increasing vascular cell adhesion protein1 (VCAM1) and NAD-dependent deacetylase sirtuin-1 [73]. An impressive number of CMV-encoded miR were also identified [74] and it was postulated that they can also participate in vascular remodeling and endothelial injury [50]. A summary of some pro-atherosclerotic CMV-encoded miRs according to cell type function regulation is described in Table 3.

Table 3. CMV encoded miR according to cell type function in atherogenesis.

CMV Encoded miR	Cell Type	Function	References
miR-217	Endothelial	Angiogenesis	[50,75]
miR-US25-1	Endothelial	Ox-LDL induced apoptosis	[76]
miR-UL112	Endothelial	Endothelial dysfunction by modulation of multiple signal pathways	[77,78]
miR-US4-5p	Macrophages	Apoptosis	[50,79]
miR-US33	Vascular smooth muscle cells	Apoptosis	[80]

3.4. Animal Model Studies

Due to its species specificity, human CMV in-vivo studies are quite limited and animal models are frequently used to mimic genes/proteins and the secretome of human CMV. Of all animal models, rat and murine models have the highest homology to human CMV [81,82]. Rat and murine infection models produce acute as well as latent infection, with an important symptomatology in immunosuppressed animals. In murine models of atherosclerosis, CMV promotes a rapid course of transplant vascular sclerosis and induces upregulation of a series of genes involved in both angiogenesis and wound repair [83] and promotes an increased cytokine expression in the infected aortic samples [84]. CMV viral transcripts can modulate the host immune response and apoptosis [71], promoting endothelial damage and thus, atherosclerosis. For example, UL122 expressed on endothelial cells during early stages of infection show homology to connexin 45 and integrin alpha 3 and 6, while US28 also expressed on endothelial cells surface shows homology to integrin alpha 6 (CD49f) and seems to be involved in CMV reactivation from latency [68]. CMV IE2 gene delays smooth muscle cells apoptosis by upregulating the expression of antiapoptotic proteins Mcl-1 and Bcl-2 and was linked to a myocardin-induced transcriptional program responsible for aortic smooth muscle cells proliferation in rats' aortas [85]. Furthermore, the role of CMV in cardiovascular disease seems to extend from a possible risk factor for atherosclerosis to direct myocardial pathology, as a recent animal model study demonstrates direct murine CMV infection of myocardial cells, with tachycardia and hypertrophy, suggesting CMV reactivation within the heart and vasculature [86]. The same murine model study showed viral DNA persistence within myocardial cells up to 100 days post-acute infection, but with a viral gpB gene expression of only 35 days post-infection, suggesting that CMV latency may start within the heart after acute infection [86]. Murine CMV has been associated with myocardial fibrosis, synus tachicardia and ventricular hypertrophy in chronically infected mice [87]. The results of these animal studies are additional proof for a possible role of CMV infection in atherosclerosis, cardiovascular disease, and direct myocardial injury.

4. Conclusions

According to the World Health Organization, cardiovascular disease is the leading cause of death worldwide. Despite all efforts in preventing the conventional risk factors, the number of deaths related to myocardial infarction, stroke, peripheral artery disease,

or hypertensive pregnancy disorders is increasing. The involvement of infectious agents in the mechanisms of atherosclerosis has been studied for more than two decades. The association between CMV, atherosclerosis, and cardiovascular diseases is still controversial and needs to be clarified. Due to its ability to infect almost all cell types and its secretome largely based on proinflammatory cytokines, chemoattractant, and vascular growth factors, CMV triggers plaque formation by a complex mechanism of endothelial injury, alteration of lipid metabolism with lipid deposition, vascular smooth muscle cells proliferation and migration, as well as disruption of coagulation mechanism and thrombosis.

This review highlights that CMV infection may be associated with acute atherosis, atherosclerosis, and/or cardiovascular disease in almost all population groups. It may produce vascular injuries eversince placentation and then continue to maintain a chronic inflammation status with subsequent vascular impairment and cardiovascular events later in life, in both healthy and immunosuppressed patients. Direct detection of CMV's DNA or antigens in atherosclerotic patients is the best proof for a possible role of this ubiquitous virus in plaque development. In order to answer if there is a role as an independet risk factor for CMV in atherosclerosis and cardiovascular disease, complex multicentric cohort studies enrolling newborns, children, adolescents, young adults, adults, and elderly should be considered. This would allow a better understanding of the role played by chronic CMV infection and reactivation in atherogenesis and cardiac disease, in a very exciting era, in which new therapeutic and prophylactic strategies including vaccination are already in progress for this infectious agent.

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CHAPTER 3. PROJECT AIMS

Romania has one of the highest mortality rates due to cardiovascular causes among the European Union states. The main cause of death is myocardial infarction followed by stroke. The population's lifestyle and a deficient medical system contribute to this leading position in the European ranking [120,143]. The public health programs developed from 2000 until now led to a slight improvement in life expectancy, but cardiovascular disease remains the main cause of death in Romania. The possible association of a infectious cause in the initiation and development of atherosclerotic lesions has been largely debated, with HCMV infection being the main potential trigger identified in large studies. The seroprevalence of HCMV infection varies between 30 and 80% in the general population in Europe, depending on the socio-economic level. Latent CMV infection remains largely asymptomatic in immunocompetent individuals, but can elicit fulminant cardiovascular, pulmonary and neurological manifestations of varying severity, in immunocompromised individuals, (HIV-infected and transplanted patients), through multiple reactivations.

Thereby, the main goal of this study was to evaluate a possible connection between CMV infection and the early development and progression of cardiovascular diseases. The specific objectives were:

1. To evaluate the cytomegalovirus seroprevalence in the Romanian population, in different age and risk groups, and to investigate a possible correlation between anti HCMV antibody titers, as an expression of multiple viral reactivations and the cardiovascular risk.
2. To assess the dynamic action of HCMV on the vascular wall, using the expression of proinflammatory cytokines and growth factors, in special risk groups, such as patients who developed preeclampsia during pregnancy, due to the emerging evidences on an increased risk of progression towards cardiovascular disease in this particular risk group [4,12,136]. To gain a mechanistic view on this pathology, in collaboration with the National Reference Center for Cytomegalovirus in Limoges, France, we set out to investigate particular aspects related to HCMV-mediated vascular remodeling and the expression of various genes with a role in atherosclerosis within the placenta.

CHAPTER 4. HCMV IGG SEROPOSITIVITY AND ATHEROSCLEROTIC CARDIOVASCULAR DISEASE SEVERITY IN A ROMANIAN COHORT

4.1 Background

The European mortality due to cardiovascular disease has been constantly increased during the last decade. The distribution of death between the European Union countries is highly influenced by population lifestyle, different ethnic genetics, or socio-economic status. Cardiovascular related death ranking in the EU is presented below in figure 8.

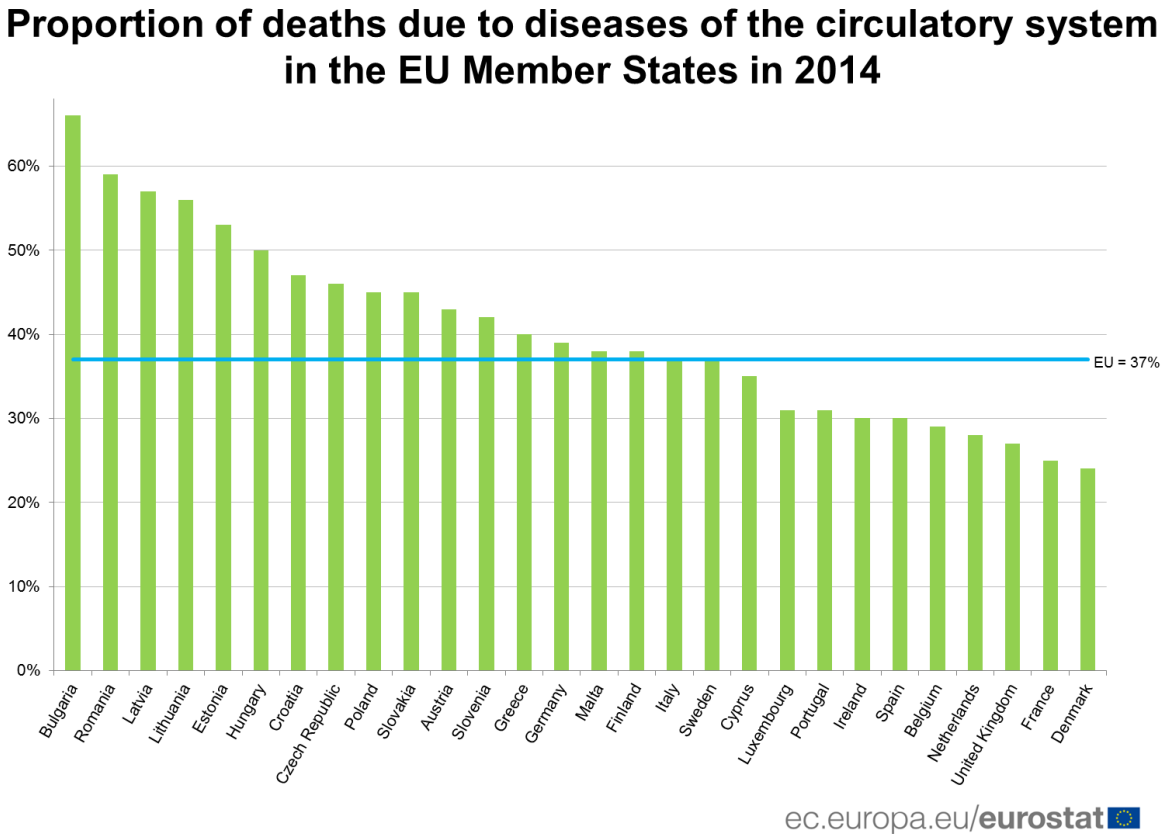


Figure 8. Mortality from cardiovascular causes in the European Union

https://ec.europa.eu/eurostat/web/products-eurostat-news/-/edn-20170928-1

Despite the introduction of several prevention programs, the incidence of common cardiovascular risk factors such as hypertension, dyslipidemia, obesity, diabetes, or smoking is still high in Romania [131]. There are many vulnerable groups with an increased early risk of cardiovascular diseases, including the long term survivors of the HIV pediatric epidemic [75,79], who were infected parenterally during the late ‘80s-early ’90. These patients lived with HIV infection all their childhood and have an increased risk

of an early development of atherosclerosis. There are several studies pointing out an increase prevalence of other viral infections such as hepatitis B and herpesviruses in this particular cohort. Despite all this background and the clear evidence of association between HCMV and cardiovascular disease worldwide, there are very few published studies on HCMV seroprevalence in Romania. In addition, to our knowledge, to date, no other research focused on the possible association of this infection to cardiovascular disease in Romania. The data available up to this point provide unicentric information about HCMV seroprevalence in pregnant women, this being estimated at approximately 95% among women of childbearing age [58,66].

4.2 Materials and methods

A total of 422 patients aged 19 to 79, mean age 47.79 years old were enrolled in this study. Of these, 228 patients, mean age 22.15 years old, were included retrospectively from an HIV-positive cohort, available at the Stefan S Nicolau National Institute of Virology in Bucharest, Romania. This cohort included HIV treated patients admitted between 2012-2016 from several departments of infectious diseases in Bucharest. The rest of 194 patients, mean age 55.5 years old, were prospectively enrolled from general population, from the internal medicine department of the Coltea Clinical Hospital in Bucharest, Romania, between 2016-2017. To carry out this study, all legal and ethical provisions in force were respected. All patients included in this study signed a written consent form, agreeing to the collection of blood samples as well as data from their medical records and their processing for research purposes. The data obtained from all 422 patients were used exclusively for questioning HMCV seroprevalence and studying the distribution of this infection in the HIV positive population and the general population. This part of our research was partially funded by an internal grant project, PFE_23/2018, from the University of Medicine and Pharmacy "Carol Davila", Bucharest. For the prospective cohort some inclusion and exclusion criteria were selected as pictured in table 2. In addition to seroprevalence, this cohort was also used to assess the association of the anti-CMV IgG antibody titer with left ventricular hypertrophy and the severity of cardiovascular disease.

Table 2. Inclusion and exclusion criteria for the seroprevalence prospective cohort

	INCLUSION CRITERIA	EXCLUSION CRITERIA
	• Age 18-65 years old	• Actual diagnosis or history of:

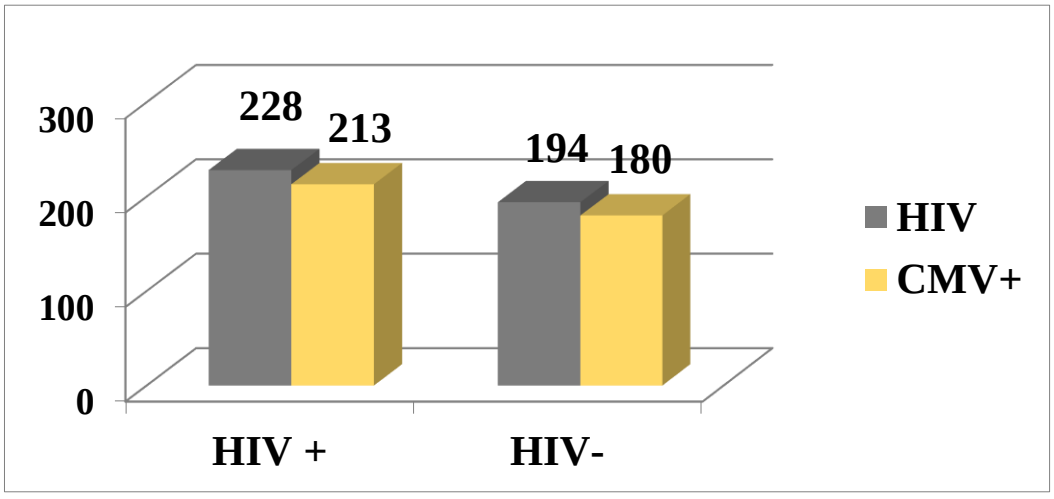
PATIENTS	• Known cardiovascular disease • Unknown cardiovascular disease • Urban and rural environment	✓ Neoplasia ✓ Autoimmune disease ✓ HVB or HVC infection ✓ HIV infection ✓ Epstein Barr infection ✓ Sepsis

The presence of anti-HCMV IgM and IgG antibodies was assed for all patients included in this study at Stefan S Nicolau National Institute of Virology, Bucharest, Romania, using a quantitative enzyme immunoassay (DiaPro, Diagnostic Bioprobes SRL, Italy). ELISA screening tests for HIV were also performed in all patients. For HIV positive cohort the quantitative determination of viral nucleic acid by RT-PCR was also available. The obtained data were processed statistically with the help of Windows 10 office Excel and R software 4.1.1. For the prospective cohort more detailed materials and methods were published in an original article – see section 4.5.

4.3 Results

Seroprevalence of latent HCMV infection, highlighted by the presence of specific IgG anti-HCMV antibodies was comparable in general population and positive HIV patients. There were no statistical differences, between the two groups, 92,7% in HIV positive patients vs 92,9% in HIV negative patient, p value 0.93 (figure 9).

Figure 9. CMV seroprevalence in HIV positive population versus HIV negative population



The average titer of specific IgG HCMV antibodies was higher among HIV+ patients compared to HIV- participants (average concentration: 8.76 iu/ml vs. 4.85 iu/ml, p= 0.06). In the HIV positive group, subjects with higher titers of IgG HCMV antibodies (> 8.76 IU / ml) had significantly lower values of the current number of CD4 cells (367 vs. 523, p = 0.02), of the CD4 /CD8 ratio (0.39 *vs* 0.74, p = 0.01) and of the number of CD4 nadir (45 vs 143, p = 0.003), as well as significantly higher values of zenith HIV RNA (5.3 vs. 4.2 copies/ml, p = 0.001) (figure 10). There was no significant correlation between cardiovascular risk parameters (serum triglycerides, cholesterol, blood glucose, blood pressure values) and the level of anti CMV antibodies. Unfortunately, assessment of the left ventricular function or evaluation of the intima-media thickness was not available to assess an early increased risk of atherosclerosis. A prospective study looking for these aspects will bring additional important information on the clinical risk for cardiovascular diseases in this vulnerable group of patients.

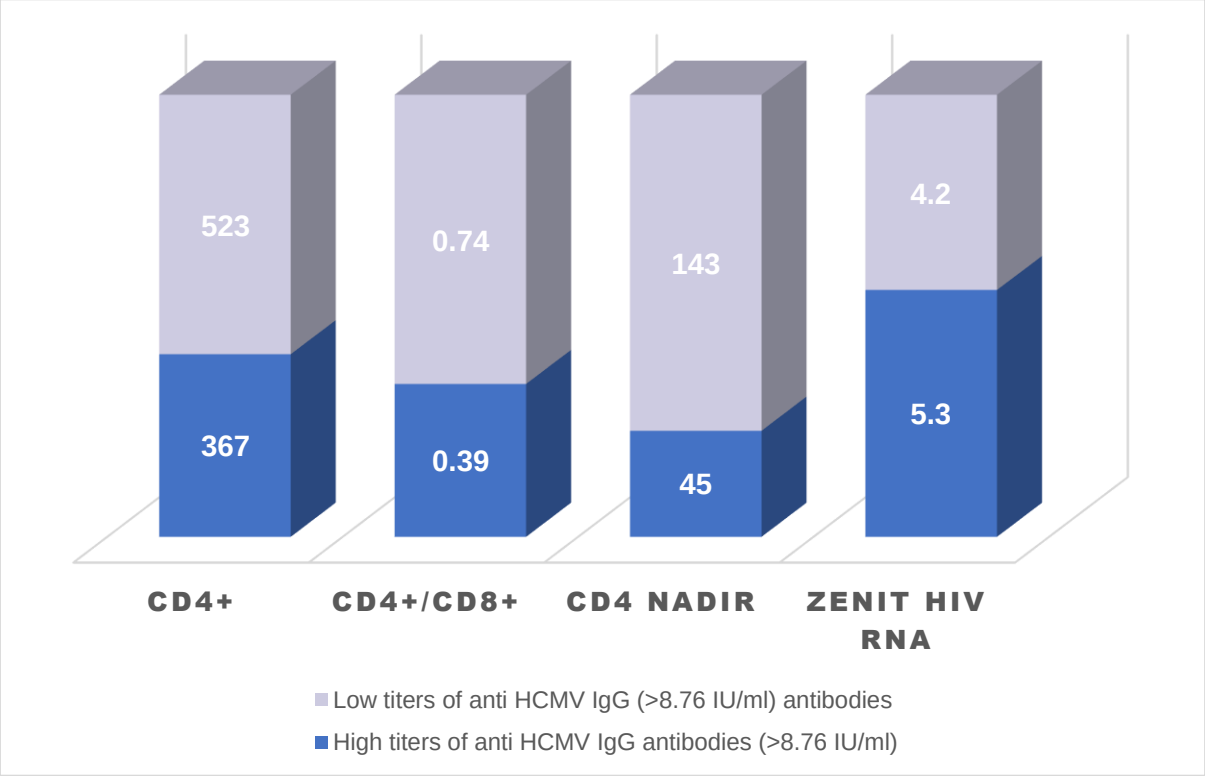


Figure 10. The expression of different types of markers in HIV monitoring in patients co-infected with HCMV

In the subgroup of HIV negative patients, the assessment of left ventricular hypertrophy was performed and correlated with the HCMV seroprevalence. The detailed results of the association of HCMV infection with left ventricular remodeling and with the severity of

cardiovascular disease, as well as the importance of this infection as a single risk factor or in association with other cardiovascular risk factors were published in an original article – see section 4.5.

4.4 Discussions

Our data suggest a very high prevalence of HCMV infection in Romania of more than 90%. However, much larger multicenter cohort studies are needed to confirm the previously reported numbers. In Romania and not only, the use of modern therapy for HIV infection like cART, drastically changed the rate of mortality and morbidity associated with HIV, but large cohort studies on patients living with HIV show an increased risk for cardiovascular disease and cardiovascular events when latent HCMV infection is associated [95]. Of all cardiovascular entities, ischemic heart disease followed by stroke are the most incriminated causes of death in this particular group of patients [34,95]. cART therapy itself is considered as an aggravating factor for atherosclerolerosis in many studies [46,84] as it has a strong metabolic action causing dyslipidemia, diabetes or hypertension [84]. Thus, cART therapy together with HCMV infection in HIV infected patients play a key role in atherosclerosis development and progression to cardiovascular disease [95,155,162]. The HIV seroprevalence infection in Romania in 2021 as reported for group age 15-49 years old is less than 1% [180]. However, the majority of people parenterally infected during the HIV pediatric epidemic are towards the upper limit of this interval or slightly outside of it or have already been treated for more than 20 years. Considering this context, it would have been interesting to have clinical data for HIV/HCMV coinfected patients to compare the severity of cardiovascular disease to a non-HIV infected group. In our prospective cohort – results published in an original article, section 4.5 - the mean age was above 55 years old the severity of the cardiovascular disease was strongly associated to higher titers of anti HCMV IgG antibodies. However, a very small percentage of pacients, 5% of HCMV positive man had an acute myocardial infarction. No patient in our prospective cohort suffered from stroke. Further Romanian populational studies on larger cohorts of patients are needed to clarify the hypotheses issued on this project. We consider that one of the biggest biases of our study is represented by the absence of two very important population groups, namely children and the elderly. Some comparative seroprevalence data between Romania and France as well as french territories are presented in section 4.6 as a paper in progress for submission. For a better assessment of risk factors and severity of cardiovascular disease comprehensive

studies need to be conducted in Romania, a leading country of cardiovascular death, where HCMV infection seroprevalence is higher than 90%.

4.5 Original article

https://farmaciajournal.com/issue-articles/cytomegalovirus-infection-and-cardiovascular-risk-in-a-monocentric-romanian-adult-patient-group/ [33]

https://doi.org/10.31925/farmacia.2022.2.16 **ORIGINAL ARTICLE**

CYTOMEGALOVIRUS INFECTION AND CARDIOVASCULAR RISK IN A MONOCENTRIC ROMANIAN ADULT PATIENT GROUP

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Abstract

Cytomegalovirus (CMV) is a widely distributed herpes virus in human populations that rarely causes any symptoms to healthy individuals. The infection can lead to serious health complications and even death for immature and immunocompromised people. Multiple studies have suggested that CMV could be a risk factor for developing cardiovascular diseases even in healthy carriers. Atherosclerosis is the root cause of many diseases like stroke or cardiovascular-related pathologies like angina, ischemic heart disease, acute myocardial infarction and cardiovascular death. In this study, we aimed to demonstrate the link between CMV serostatus and cardiovascular parameters like left ventricle mass and cholesterol level in a cohort of 145 volunteers. Also, we researched the benefits of cholesterol-lowering drugs in the prevention of CMV induced atherogenesis. The study revealed that the severity of cardiovascular disease is associated with an increased CMV IgG titter.

Rezumat

Citomegalovirusul (CMV) este un virus herpetic răspândit pe scară largă în rândul populației umane, care rareori provoacă simptome persoanelor sănătoase. Infecția poate duce la complicații grave de sănătate și chiar la deces pentru persoanele imature și imunocompromise. Mai multe studii au sugerat că CMV ar putea fi un factor de risc pentru dezvoltarea bolilor cardiovasculare chiar și la purtătorii sănătoși. Ateroscleroza este cauza principală a multor boli, cum ar fi accidentul cerebral vascular sau patologii cardiovasculare precum angina pectorală, boala cardiacă ischemică, infarctul miocardic acut și decesul cardiovascular. În acest studiu, ne-am propus să demonstrăm legătura dintre serostatusul CMV și parametrii cardiovasculari, cum sunt masa ventriculului stâng și nivelul colesterolului într-o cohortă de 145 voluntari. De asemenea, am cercetat beneficiile medicamentelor care scad colesterolul în prevenirea aterogenezei induse de CMV. Studiul a arătat că severitatea bolilor cardiovasculare este asociată cu un titru crescut de IgG-CMV.

Keywords: Cytomegalovirus infection, cardiovascular disease, diabetes, atherosclerosis

Introduction

Cardiovascular disease is a global health burden regardless of all efforts in reducing risk factors like hypertension, smoking, high LDL-cholesterol levels, diabetes or obesity [36]. Nowadays, it is well-known that the main underlying process of cardiovascular disease is atherosclerosis, with chronic arterial wall inflammation as the key process in initial and progressive atherosclerotic lesions [32]. During the last decades, many infectious agents that may cause an arterial inflammatory status were investigated [9, 10, 23]. Of them, cytomegalovirus is the only one constantly linked to atherosclerosis, cardiovascular disease and vascular neurocognitive disorders [1, 12, 21, 22].

Human cytomegalovirus, a member of the *Herpesviridae* family, is highly prevalent worldwide. According to World Health Organization, CMV infection is a major cause of disease and death in immunocompromised people and the leading viral cause of congenital disabilities in the world [14].

CMV is a ubiquitous DNA virus, with a worldwide seroprevalence from 80% to 100%, preponderantly depending on the socio-economic status [2]. According to CDC, in the US, over 50% of adults will develop the infection by the age of 40. Also, a third of the children

younger than five years old are already infected with CMV [11].

Using various immune evasion mechanisms, CMV can establish latency in almost all types of cells and tissues [8]. Therefore, it has been linked to all stages of atherosclerotic lesions, from endothelial injuries to atherothrombotic events [1, 4, 13].

The virus was detected in the atherosclerotic plaque and stimulates the pro-inflammatory signals in the cell environment promoting activation of both endothelial cells and peripheral blood mononuclear cells (PBMC), increasing their susceptibility to infection and cardiovascular risk [5, 22, 38].

The cholesterol-lowering drugs like statins may be important during treatments for reactivations of CMV infection, especially in the elderly and patients with other associated diseases like diabetes, in order to decrease the associated cardiovascular complications [16, 34].

Moreover, severe cardiovascular adverse events are decreased by 50% in diabetic patients under the treatment with statins due to their pleiotropic effects including lipid-lowering, anti-inflammatory, antioxidant, antithrombotic and antimitotic effect [27].

The study aims to highlight the impact of chronic cytomegalovirus infection on health status and assess the cardiovascular risk in chronically infected patients and the importance of monitoring these patients for initiating the optimal therapeutic approach.

Materials and Methods

Study design

One hundred and forty-five patients, aged 20 to 79 years old, mean age 55.13 years, admitted to the Department of Internal Medicine of Colțea Clinical Hospital in Bucharest, Romania, from February to December 2016, for acute or chronic pathologies, with a known or unknown cardiac disease were enrolled in the study. All patients gave informed consent, and the study was approved by the Ethics Committee of Colțea Clinical Hospital. At enrolment, each patient filled out a questionnaire on risk factors for cardiac disease (cigarette smoking, alcohol, diabetes, obesity, serum cholesterol levels and family risk factors) and medication use. The inclusion criteria were the confirmed presence of chronic infection with cytomegalovirus. The exclusion criteria were paediatric population and actual diagnosis or history of neoplasia, autoimmune disease, hepatitis B or C virus infection, HIV infection, Epstein Barr virus infection and sepsis.

All patients carried out a physical examination with arterial blood pressure measurement, cholesterol profiles evaluation using an Ortho Clinic Diagnostics automated dry biochemistry analyser and cardiac ultrasound evaluation using a Siemens Acuson X150 ultrasound device.

Left ventricular mass (grams) and left ventricular mass indexed to total body surface area were determined using the Devereaux modified formula:

$$0.8\{1.04[([LVEDD + IVSd + PWd]3 - LVEDD3)]\} + 0.6.$$

The severity of cardiac disease was assessed using AHA and ESC guidelines as well as New York Heart Association (NYHA) functional classification [26]: 0 = no cardiac changes; 1 = light cardiac changes – NYHA I; 2 = mild cardiac changes – NYHA II; 3 = moderate to severe cardiac changes – NYHA III – NYHA IV; and 4 = recent history or acute indication of minimally invasive or surgical procedures for cardiovascular disease.

Biochemical assays

Six mL of venous blood in a clot activator tube and 6 mL of venous blood on EDTA were collected from each patient. Anti-CMV IgG and IgM levels were measured in the "Ștefan S. Nicolau" Institute of Virology, Bucharest, Romania, using a quantitative enzyme immunoassay (DiaPro, Diagnostic Bioprobes SRL, Italy). According to the manufacturer's instructions, a sample with a reactivity over 0.5 UI/L was considered positive. The patients were divided into two groups based on the median value of reactivity for the CMV positive samples = low CMV IgG titters (0.5 - 5 UI/L) and high CMV IgG titers (> 5 UI/L).

Statistical analysis

Statistical analysis was implemented using the open-source software R (R version 4.1.1.) [29]. Numerical data sets were summarized by descriptive statistics, standard deviation and 95% confidence intervals. Bootstrapped Welch two-sample t-test was used to analyse the difference of the two numerical data sets not normally distributed [3]. The categorical variables were analysed by calculating percentages (%) and assessing associations with Pearson's chi-square test or by Fisher's exact test when the assumptions were not met. A part of the numerical data sets was transformed into qualitative variables (such as Age and BMI) and expressed by percentages (%). Statistical significance level was considered at alpha 5% ($p < 0.05$).

Results and Discussion

The study included 145 patients (43% male, 57% female) (Table I). All patients had a positive IgG anti CMV antibodies status. No acute CMV infection using IgM anti-CMV antibody status was detected. 25% of patients were under 50 y.o., 64% were 50 - 65 y.o. and 11% were over 65 y.o. 60% of patients presented at least light cardiac changes (Figure 1a), and 28% had diabetes (Figure 1b, 15% of them were insulin-dependent). 33.7% of patients were active smokers, and of them, 59% were heavy smokers with a consumption of more than 30 pack-year. 3.4% of patients declared chronic alcohol consumption of 60 to 100 g of alcohol/day.

Table I

Patients diagnosed with cytomegalovirus (CMV) infection

		Chronic CMV infected patients (n = 145)	
		Male % (n = 62)	**Female % (n = 83)**
Age	< 50 y.o.	26%	24%
	50 - 65 y.o.	65%	64%
	> 65 y.o.	10%	12%
Cardiac changes	0	35%	42%
	1	39%	46%
	2	6%	6%
	3	15%	6%
	4	5%	0%
Hypertension	0	37%	29%
	1	5%	7%
	2	21%	13%
	3	37%	51%
Diabetes	0	68%	76%
	1	27%	20%
	2	5%	4%
BMI	Overweight (> 25 kg/m^2)	76%	73%
	Normal weight (17 - 25 kg/m^2)	19%	22%
	Underweight (< 17 kg/m^2)	5%	5%
Smokers		45%	26.5%

Cardiovascular disease severity degree (0 = no cardiac changes; 1 = light cardiac changes – NYHA I; 2 = mild cardiac changes – NYHA II; 3 = moderate to severe cardiac changes – NYHA III; 4 = recent history or acute indication of minimally invasive or surgical procedures for cardiovascular disease – NYHA IV); Hypertension (0 = normal, 1 = grade I, 2 = grade II, 3 = grade III); Diabetes (0 = no diabetes, 1 = oral antidiabetics treatment, 2 = insulin-dependent treatment); BMI = body mass index

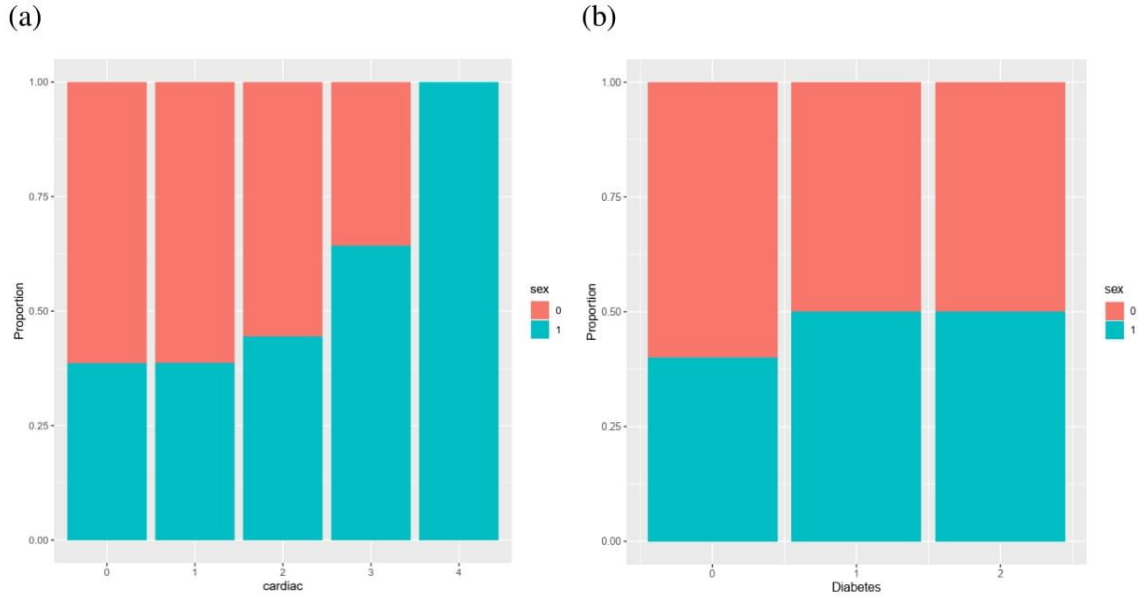

Figure 1.

Cardiovascular disorders (a) and diabetes (b) according to gender in patients with CMV infection (0 = female and 1 = male)

Most of the patients with cardiac changes ($p > 0.05$) and diabetes ($p < 0.01$) in our study were overweight, a condition that brings extra pro-inflammatory status and oxidative stress in the context of chronic CMV infection (Figures 2a and 2b).

CMV serostatus was assessed by determining the level of specific IgM and IgG antibodies in patients' blood. No patient presented IgM anti-CMV antibodies. Patients with a level of IgG antibodies over 0.5 UI/L were considered positive for chronic CMV infection. The patients were divided into two groups according to the blood level of antibodies as follows: group 1 included low anti-CMV IgG titters (0.5 - 5 UI/L), and group 2 had high anti-CMV IgG titters (> 5 UI/L).

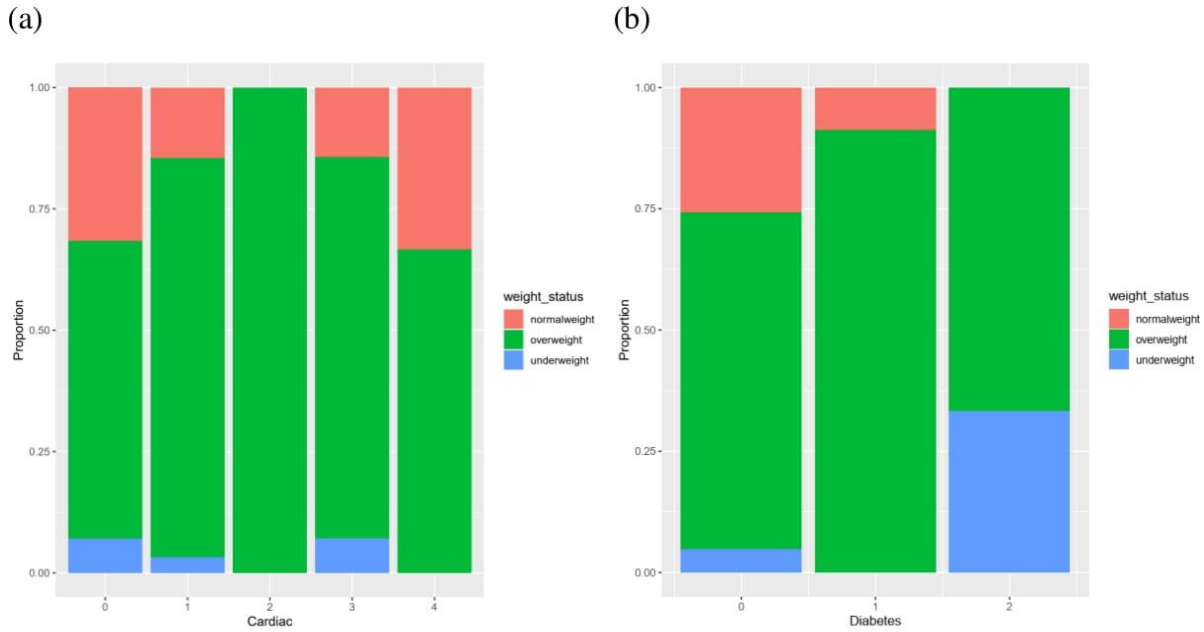

Figure 2.
Body mass index among the CMV infected patients diagnosed with cardiovascular disease (a) and diabetes (b)

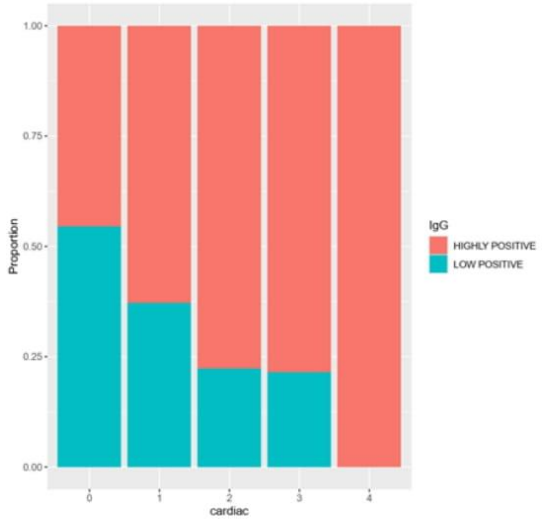

Figure 3.
The distribution of patients diagnosed with cardiovascular risk according to CMV IgG titter

We observed that the severity of cardiac disease (Figure 3) increased significantly with the CMV IgG titter ($p < 0.05$). Thus, 63% of NYHA I patients, 78% of NYHA II patients and 79% of NYHA III patients had high titters of IgG. All patients with NYHA IV cardiac failure or recent history or acute indication of minimally invasive or surgical procedures for cardiovascular disease belonged to group 2 with specific IgG titter over 5 UI/L. As for the left ventricle mass index alone and the severity of the cardiac disease in patients infected with CMV, the p-value of the test was < 0.001. As expected, the severity of cardiac disease was significantly correlated with the left ventricle mass with a correlation coefficient of 0.41, indicating a moderate to a strong relationship. Instead, we observed a low correlation (0.22) between the left ventricle mass index and specific IgG blood titter.

As a previous study showed, cardiac hypertrophy could be the consequence of long-term exposure to IL-6, an interleukin produced in larger amounts during CMV reactivation in chronic infection [7]. The reactivation of the CMV within the heart leads to altered cellular responses, which trigger the proliferation of endothelial and smooth muscle cells. The pro-inflammatory response helps CMV reactivation, causing a vicious circle that worsens the cardiovascular tissue's negative effects [6]. As previously mentioned, atherosclerosis is the main cause of cardiovascular disease, a complex process based on two key factors: inflammation and lipid accumulation within arterial intima [24]. The atherosclerosis risk is known to be higher in patients diagnosed with cytomegalovirus infection [19]. CMV infection could be an underlying cause that enhances the formation of atheroma plaque and the progression of atherosclerosis through different mechanisms, most of them aiming arterial wall inflammation. Once infected, vascular endothelial cells are prone to integrity disruption, dysfunctional metabolism and even apoptosis, a damaging process mediated by viral proteins IE84 and IE72. Moreover, antibodies antiviral proteins UL122 and US28 can activate growth factors, cytokines, and adhesion molecules, which are connected to the evolution of atherosclerosis. Usually, endothelium displays anti-atherosclerosis proprieties by minimizing thrombosis and inflammation. Yet, some research demonstrated the opposite in CMV-infected vascular endothelium, meaning a pro-coagulant response and an increased aggregation and adhesion of thrombocytes and an

upregulation of inflammatory factors like IL-6, IL-8 and RANTES [38].

Table II

The variation of blood parameters according to the presence of cardiovascular risk among CMV patients

		Mean ± SD
Total cholesterol	-CV	185.69 ± 43.7
	+CV	189.37 ± 53.8
HDL - cholesterol	-CV	52.95 ± 25.4
	+CV	47.41 ± 14.3
LDL - cholesterol	-CV	110.76 ± 35.4
	+CV	110.11 ± 44.4
Triglycerides	-CV	111.36 ± 65.8
	+CV	160.26 ± 77.8*

-CV/+CV = absence/presence of the cardiovascular risks; * P (Wilcoxon) < 0.05

To achieve both key factors in atherosclerosis, we also evaluated the blood lipid profiles of all patients included in our study (Table II).

In the case of total cholesterol, HDL-cholesterol and LDL-cholesterol blood levels, there were no significant statistical differences between the patient group with at least one cardiovascular risk and the group with no cardiovascular risk reported (Table II). Still, in both groups, the LDL-cholesterol blood levels were higher than 75 mg %, which was a concerning aspect, especially for the patients with active CMV infection and already installed cardiovascular disease.

The treatment with statins was considered for 41 patients included in the study. The blood level of total cholesterol ($p < 0.01$) and LDL-cholesterol ($p < 0.01$) was significantly decreased among patients treated with statins compared to those who did not receive cholesterol-lowering medications (Figure 4).

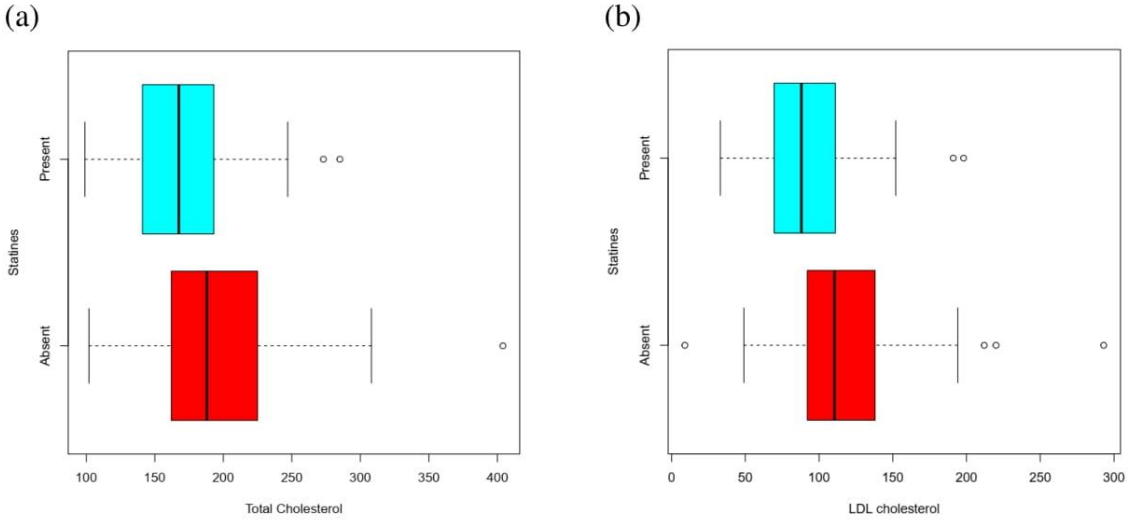

Figure 4.

The impact of the statins treatment on the blood level of total cholesterol (a) and LDL-cholesterol (b) in patients diagnosed with CMV infection

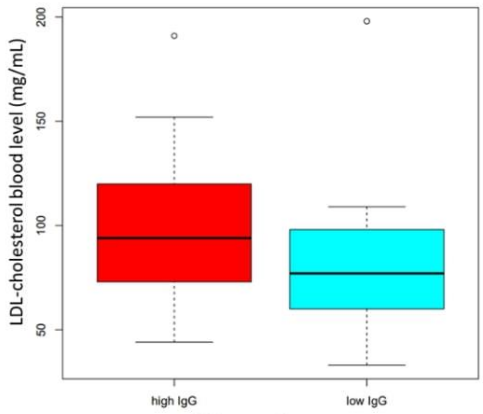

Figure 5.

LDL-cholesterol level according to IgG CMV antibodies titter (high IgG > 5 UI/L, low IgG < 5 UI/L) in patients treated with statins

In the case of the treatment with statins, the CMV infected patients with a high titter of IgG antibodies showed a slight increase of mean LDL-cholesterol level but with no significant differences compared to the patients with low IgG CMV titters (Figure 5). The treatment with statins, used primarily for their hypolipaemiant role, has been shown through different studies also having beneficial effects on reducing CMV titters in endothelial cells [16, 17, 34]. Comparable with ganciclovir antiviral activity, statins were potent also on ganciclovir-resistant CMV strains [28]. The antiviral activity was observed in atorvastatin, simvastatin and fluvastatin, which seems to curb the replication chain of the virus by reducing the formation and activity of CMV's IE antiviral protein antigens [23].

During the acute infection, CMV targets the endothelial cells, smooth muscle cells and the immune system cells. CMV is transmitted by blood and contaminated

secretions (saliva and urine) *via* intercourse, organ transplantation, transplacental or through lactation from mother to new-born [33]. Once infected, a healthy organism is never cured of CMV. The virus persists as a latent infection and can reactivate anytime if the immune system is impaired [31]. The virus is kept latent mainly due to the action of T-cells, but other immune cells like NK, monocytes and macrophages are also involved [20]. CMV infection acts through inflammatory and immunological mechanisms. It has been stated that cytomegalovirus affects the host's immune system as no other existent human pathogen in terms of latency and enlargement of immune cells populations. The immune system reacts by boosting the number of memory T cells phenotypes and NK cells for CMV-seropositive persons. This phenomenon is then followed by inflammatory and cytotoxic processes [30]. Depending on the period of life when a person acquires CMV, the virus can have both: favourable or deleterious effects on the host's immunity. Intrauterine infection usually ends with life-long neurologic squeals for new-borns and it was even associated with chances of developing acute lymphoblastic leukaemia later in life. For children and young adults, it was demonstrated a correlation between CMV-seropositivity, a better response to influenza vaccine and protection against other pathogens [30]. For elderly people, the presence of CMV in their system is mostly associated with cardiovascular disease and less with other pathologies like infections and cancer [25]. The long-term infection itself could lead to the production of pro-inflammatory cytokines like IL6 and TNF-alpha, which are also mortality related factors. A large titter of antibodies may result from the multiple reactivations of the CMV, which is, in fact, a path to a much more important inflammatory response that could damage vascular endothelium and the cardiovascular system overall [35].

Multiple studies have been suggesting that CMV could be a risk factor for the development of cardiovascular diseases, especially in the progress of atherosclerosis. Some studies did not succeed in showing any relationship between the presence of CMV in vascular endothelium and atherosclerotic plaque and almost no difference when compared the CMV in healthy individuals' vascular cells with those who have atherosclerosis [37]. Other studies, on the contrary, demonstrated a higher replication of the virus in the atherosclerotic plaques for those with acute coronary disease and a link between familial history for cardiovascular syndromes and the spreading of CMV in the vascular endothelium [18]. Besides atherosclerosis, CMV is suspicious as being a risk factor for the development of arterosclerosis and its negative effects like lower flexibility of arteries, elevated pulse wave velocity and high blood pressure, especially because all these are associated with an increased proportion of CMV specific T cells [25].

CMV can also stimulate other pathogens' effects. HIV/CMV coinfection is the leading cause for CMV retinitis and other serious non-AIDS pathologies, including gastrointestinal, neurological, hepatic syndromes etc. The coinfection is very common and associated with increased inflammation. Patients under antiretroviral therapy seem to have better therapy outcomes and resistance to adverse effects if they are only seropositive for HIV, but not for CMV [15].

Conclusions

The infection with cytomegalovirus increases the risk of cardiovascular disease and other associated disorders like diabetes among patients. The early diagnosis is important for patient health status evolution. The administration of cholesterol-lowering medication for an LDL-cholesterol target < 75 mg/mL should be considered for CMV infected patients in order to reduce arterial wall inflammation and regulate LDL-cholesterol homeostasis as well as to specifically control anti-CMV antibodies titters.

Conflict of interest

The authors declare no conflict of interest.

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4.6 Seroprevalence paper in progress for submission

HETEROGENEOUS REPARTITION OF HCMV SEROPREVALENCE IN FRANCE TERRITORIES AND ROMANIA

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Introduction

Cytomegalovirus (CMV) is the main cause of congenital viral infection (1). CMV primary infection during pregnancy is at higher risks of transmission to the foetus but CMV congenital infection may also occur after a non-primary maternal infection (reinfection or reactivation) in women already immunized. Cytomegalovirus seroprevalence is very variable depending from the geographical zone concerned. It is widely recognized that seroprevalence is higher in low incomes countries (from 80 to 100%) and around 50% in high incomes countries (2). In these latter, seroprevalence could vary depending from a group, a community or the region studied. Seroprevalence studies need to be conducted in countries without data such as French overseas territories or Eastern European countries, to better adapt prevention strategies.

As reflection on establishing recommendations for CMV screening is ongoing, we conducted a study with the available data for the French metropole and overseas departments and regions (DROM). We compared the CMV seroprevalence in metropolitan France, in the DROM (Réunion, Martinique, Guadeloupe and New Caledonia) and added data collected in Romania where survey is not available. In the context of this study, we could develop a closer collaboration with the overseas hospital centres and university hospitals, in order to improve our network for CMV infection surveillance and control.

Materials and methods:

Serological data collection

For metropolitan France, 35,793 results of CMV serology were collected in the Limoges University Hospital (UH) between 2008 and 2017. After excluding the duplicates, we obtained 1,139 patients between 1 and 17 years old, a population of 1,264 patients getting systematic CMV screening with serologic test outside the context of a CMV infection (282 bone marrow donors, 353 patients waiting for a kidney transplant, 393 brain-dead patients and 253 healthy volunteer subjects), 2,304 pregnant women and 3,337 serologies from patients over 65 years old. To validate this strategy of data collection, 358 samples of serum collected in pregnant women in 2015 outside of an infectious setting were selected from the biobank of the Mother-Child hospital in Limoges to perform CMV serology. In parallel of the serological data collection in Limoges Hospital, we collected data for the DROM. For Réunion Island, we could study two categories of population: 371 samples from the general population collected for other serological studies about the prevalence of bacterial pathogens in the island (CopanFLu cohort, 2009) and 2,766 patients from Saint-Denis University Hospital between January 2012 and December 2016. For French Antilles, we collected the results of 3,779 patients from the Pointe-à-Pitre UH in Guadeloupe: and of 4,465 patients from the UH in Martinique between January 2012 and December 2016. For New Caledonia, we collected data of 1,909 patients from the Nouméa Hospital

between January 2012 and December 2016 (Table 1). In Romania, 721 results were obtained from the UH of Bucarest.

Table 1: Repartition of the study population on the French territory

Population studied	Metropolitan France	Réunion Island	Guadeloupe	Martinique	New Caledonia	Total
General population	—	—	—	—	1,909	1,909
Children 1-17 years old	1,139	509	19	555	—	2,222
Adults	3,568	2,567	3,690	3,910	—	13,735
Elderly patients ≥65 years old	3,337	61	—	—	—	3,398
Total number	8,044	3,137	3,709	4,465	1,909	21,264

Methods

CMV serologies in Limoges UH were performed with Enzygnost CMV IgG (Siemens Healthcare) before 2016 on a Quadriga Befree instrument and with Liaison CMV IgG, Diasorin on a Liaison XL automate from 2016. Serology of pregnant women from serum collected in the biobank of the Mother and Child hospital of Limoges in 2015 and all serology from French overseas departments were performed with the Architect CMV IgG, Abbott Diagnostics. Dia.Pro CMV IgG, Diagnostic Bioprobes SRL assay was used in Romania (Table 2).

Table 2 : Serological testing methods

Tests	Principle	Populations analysed
Architect CMV IgG, Abbott Diagnostics	CMIA (chemiluminescent microparticle immunoassay)	Overseas French territories + 358 sera of pregnant women in Limoges (biobank)
Enzygnost CMV IgG, Siemens Healthcare	EIA (enzyme immunoassay)	Limoges hospital database, France (before 2016)
Liaison CMV IgG, Diasorin	CLIA (chemiluminescent immunoassay)	Limoges hospital database, France (from 2016)
Dia.Pro CMV IgG, Diagnostic Bioprobes SRL	ELISA (enzyme-linked immunosorbent assay)	Romania

Statistical analysis:

Seroprevalence was defined as the proportion of individuals with IgG antibodies to CMV in a target population. The following formula was used to calculate seroprevalence: (Number of cases / population) x 100. We used the statistical analysis software MedCalc (https://www.medcalc.org/calc/) to calculate a 95% confidence interval for each result and to compare the seroprevalences of each group as well as the calculated proportions within each group for different parameters. A p-value less than 0.05 concluded to a statistically significant difference between the two rates.

We used Open epi software (Dean AG, Sullivan KM, Soe MM. OpenEpi: Open Source Epidemiologic Statistics for Public Health, Version. www.OpenEpi.com, updated 2013/04/06) to assess the representativeness of our pediatric populations and patients over 65 years of age compared to the general population (2013 Insee data) and to compare seroprevalences between them with the Chi 2 test

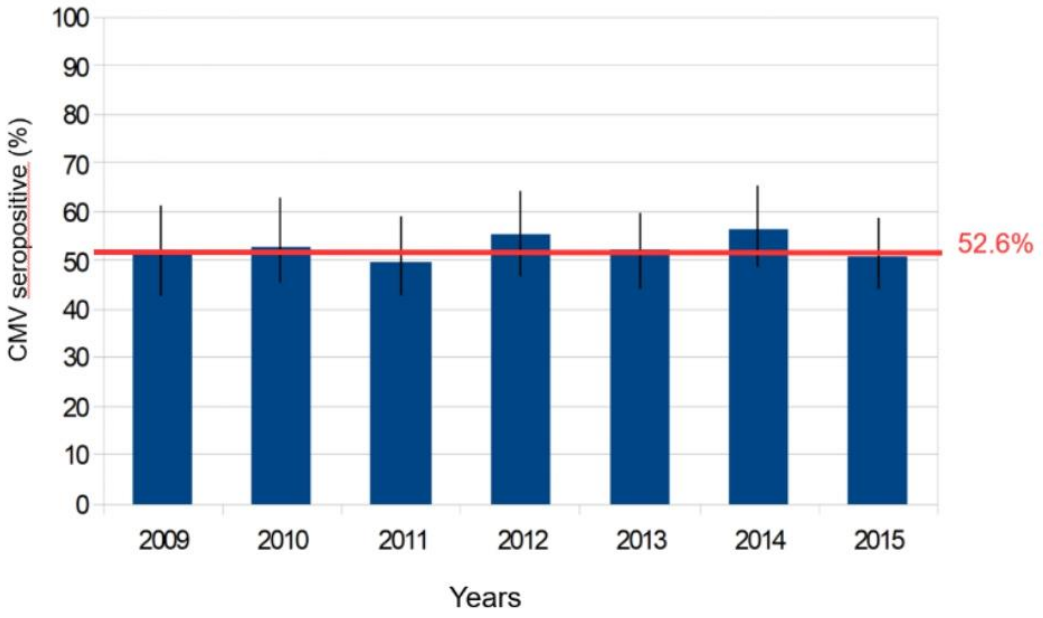


Fig.3: CMV seroprevalence in metropolitan French elderly patients

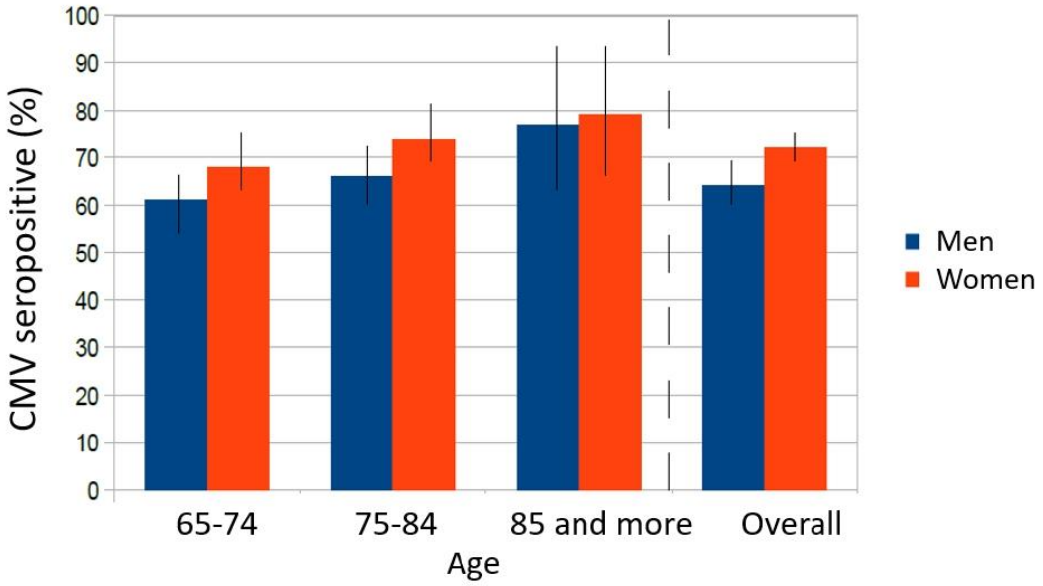


Table 3: Seroprevalence in the population with systematic CMV serology screening

North-East part of the island (81.9% vs 82.8%). Patients from the UH between 1 and 17 years of age show a seroprevalence of 62% (Fig. 5). This figure increases significantly to reach 85-90% at adult age and keeps increasing after 65 years old to 90-100% (Fig. 6).

Fig.5: **CMV seroprevalence in children from Saint-Denis Hospital :**

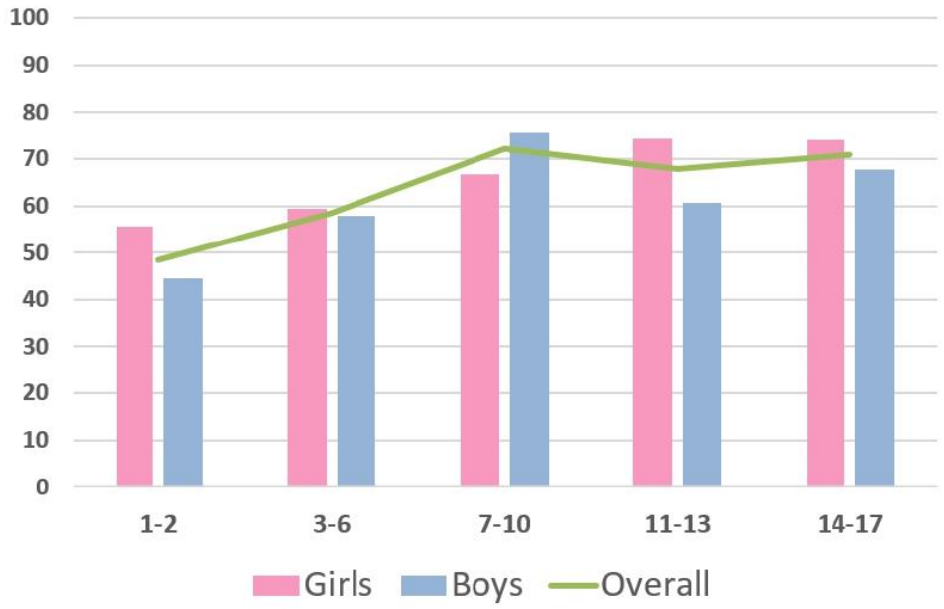
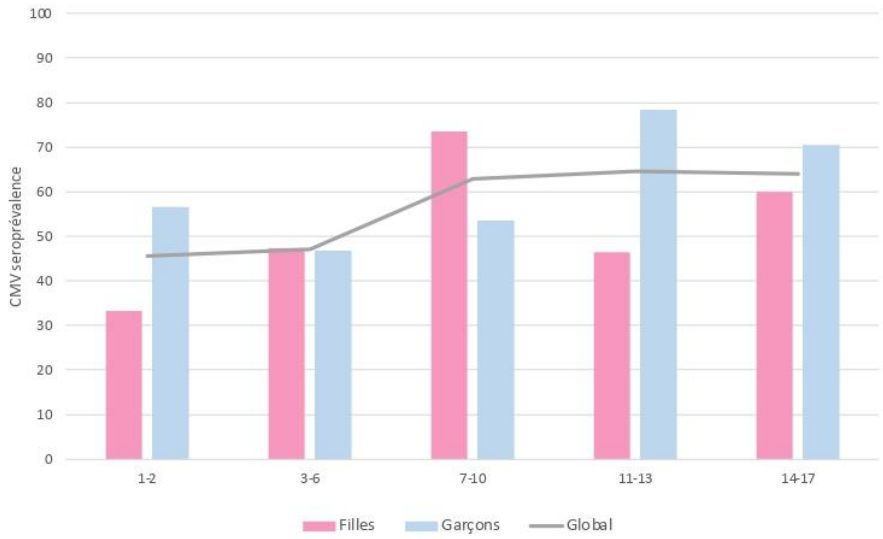


Fig.6: CMV seroprevalence in adults from Saint-Denis Hospital

Table 6: CMV seroprevalence in children in Martinique

	Children CMV seroprevalence in Fort-de-France hospital (Martinique)		
Sex	Number	% Positive	CI 95%
Male	262	53.4	44.9-63.1
Female	293	58.0	49.6-67.4
Overall	555	55.9	49.8-62.4

Fig.7: CMV seroprevalence repartition among children in Martinique



Tab.11: CMV seroprevalence in adults in Martinique

	Adults CMV seroprevalence in Fort-de-France hospital (Martinique)		
Sex	Number	Positive (%)	CI 95%
Male	1,744	89.5	87.97-90.90
Female	2,166	91.2	89.93-92.36
Overall	3,910	90.4	89.43-91.31

Seroprevalence in New Caledonia

Data from New Caledonia are similar to data from Martinique and from Réunion, with a global seroprevalence of 84.6%, and 69.2 % for patients under 17 years of age. In adults, seroprevalence is

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CHAPTER 5. HCMV AS A RISK FACTOR FOR THE ONSET AND SEVERITY OF PREECLAMPSIA IN A FRENCH COHORT

5.1 Background

As previously mentioned in section 1.4, PE is a complex, systemic medical condition that may occur in pregnancy as early as 20 weeks of gestation. The main PE characteristics are new-onset hypertension and proteinuria, although the latest American College of Obstetricians and Gynecologists guidelines recommendations no longer consider proteinuria mandatory for diagnosis [158]. This hypertensive pregnancy disorder is the leading cause of mortality in pregnant women, fetuses and newborns [139]. After pregnancy, PE was often associated to increased cardiovascular morbidity and mortality later in life [4,12]. Although the physiopathology of PE is not yet fully known, two main conditions compete for the development of this pregnancy disorder: an already altered cardiovascular status before pregnancy (such as heart failure or the association of multiple cardiovascular risk factors - most commonly obesity, hypertension, diabetes, smoking) and dysfunctional placentation [153,181]. Placental development disorders are of great interest, especially when there is lots of evidence for acute atherosis in preeclampsia [122,130,146]. Vascular remodeling of the placental arteries was mentioned for the first time by Hertig in 1945 [17], but the "acute atherosis" terminology was introduced in 1950 by Sexton and Zeek [141,169]. Interestingly, acute atherosis was never described in any other location except for the uterine vascular network [130]. Apart from the intensively studied inflammation [43,74,107], the alteration of lipid metabolism represents another important direction of research in PE and acute atherosis. For over more than 4 decades studies focused on lipid metabolism in pregnancy. The first observation is that in all pregnancies the lipid metabolism is disturbed to increase the production of lipoproteins in order to generate energy for the mother and create optimal conditions for the development of the fetus [69]. Although there is no consensus yet regarding the cut-off values of lipids in pregnancy, most of the studies consider that in normal pregnancy cholesterol values should not be higher by more than 50%, and triglycerides should not be higher than double the values measured before pregnancy, with a maximum of 250 mg/dl for total cholesterol [69]. Higher values were usually associated to bad pregnancy outcomes. When the lipid metabolism is altered, excessive oxidative stress and ROS release as a consequence of

inflammation in PE, contribute to oxLDL synthesis and accumulation within the arterial intima, leading to endothelial injury and atherosis lesion cascade activation [63,69]. Moreover, the changes in lipid metabolism during pregnancy can be transmitted to the fetus with an unfavorable impact on cardiovascular risk later in life. Thus, lipid metabolism alteration is a key mechanism for "fetal programming" hypothesis, also known as Developmental Origins of Health and Disease (DOHaD) concept [14,63]. This idea was first suggested by Barker in 1986 [23] and updated few times, until present. The concept considers that uterine environment is responsible for the newborn outcome later in life, especially in terms of cardiovascular risk [14,111].

HCMV infects the placenta and may directly and indirectly influence the fetal outcome as presented in section 2.4.3. The infection itself alters the immune response with important inflammation and it also impairs lipid metabolism. There are also lots of evidence about HCMV and cardiovascular disease. Thus, I decided to approach the HCMV infection at the level of the placenta and more precisely in preeclampsia. This part of my project was partially funded by a French-Romanian government research program – „Ion Heliade Radulescu" Scolarship, and was performed at the Inserm U1092 unit of Limoges University and at the Limoges Dupuytren University Hospital, Limoges, France.

5.2. Materials and methods

Fifty-two women of fertile age, mean age 33.15 years old (18 to 47 years old) who developed preeclampsia during pregnancy were enrolled retrospectively in this study. A control group of twenty patients with normal pregnancies, mean age 29.89 years old (22 to 37 years old) was prospectively created. All legal and ethical provisions in force were respected for this study. All patients signed an informed consent by which they agreed to the processing of their biological samples and medical record for research purposes. All patients included in this study came from the Gynecology and Obstetrics Department of CHU Dupuytren Hospital, Limoges, France. With the support of this department and that of the Pathology Department from the same hospital we collected information from patients' medical records as well as placental specimens and blood samples.

I searched the patients' ***medical record*** for: mother's age, number of pregnancies, the method of obtaining the pregnancy, any medical history before pregnancy (autoimmune diseases, HIV, hepatitis, kidney disease, any cardiovascular or oncologic pathology), cardiovascular risk factors (hypertension, diabetes, smoking, obesity, dyslipidemia), gestational age at birth, newborn's weight, infections during pregnancy, and for the test

group, the moment of the onset of preeclampsia. All data were processed using R open software 4.1.1.

Blood samples were collected from each woman during first pregnancy medical examination and at delivery. For the test group we used a sera collection from the CHU Dupuytren Mother and Child Hospital (CRBioLim biobank certified NFS96-900). For the control group fresh blood samples were used. To determine specific anti HCMV IgM and IgG antibodies a LIAISON XL automated chemiluminescence analysis was performed at the National Reference Center laboratory. All patients were also screened for HIV and hepatitis virus B and C. No HIV, nor hepatitis-infected patient was included in the study.

Placental specimens were obtained for the test group from the CHU Dupuytren Pathology collection (2016-2018) – frozen specimens kept at -80*C. For the control group, I strictly followed the collection protocol of the Pathology Department. Thus, the entire fresh placenta was collected after birth and kept at 4*C before processing (no later than 72 hours). A full thickness "carrot" biopsy was taken and immersed in liquid nitrogen for 5 seconds, then immediately transferred to dry ice and then stored in the shortest possible time at -80*C.

Genetic tests

Placental specimens were used to analyse and compare the expression of specific atherosclerosis genes (table 3). Thus, 4 different groups were designed, each of them containing three samples:

- Normal Pregnancy HCMV negative
- Normal Pregnancy HCMV positive
- PE HCMV negative
- PE HCMV positive

Each frozen sample was sectioned with a cryotome at five different levels. 20 slides of 10µm each were then stored in 1 ml of trizole, TRI Reagent® RNA Isolation Reagent for carrying out ARN extraction. After 5 minutes of dissociation 0.2 ml of cold (-20*C) chloroform was added. The tubes were vigorously stirred by hand for 15 seconds and then left at room temperature for 2-3 minutes. Afterwards, a centrifugation was performed at 11200 rpm, at 4*C, for 15 minutes. Then, the supernatant was transfered to a new tube. 600 µl of room temperature 70% ethanol was added and mixed by pipetting. Further, the mixture was transferred to a working column and the extraction continued according to the standard protocol of the QIAmp® RNA Blood Mini Kit. At the end of the extraction each

sample was tested for RNA quality using NanoDrop spectophotometer measurements and RNA electrophoresis using a standard Agilent® RNA 6000 Nano kit. The samples used for gene expression identification had a similar good RNA quality. Furthermore, cDNA was synthetized using Qiagen RT^2 First Strand® Kit. The cDNA was then amplified with the real-time RT² Profiler PCR Array (QIAGEN, Cat. no. PAHS-038Z) in combination with RT² SYBR® Green qPCR Mastermix (Cat. no. 330529). Ct values were exported to an Excel file to create a table of Ct values. This table was then uploaded on to the data analysis web portal at http://www.qiagen.com/geneglobe. Samples were assigned to controls and test groups. Ct values were normalized based on an Automatic Selection from house keeping genes (HKG) panel of reference genes. The data analysis web portal calculates fold change/regulation using delta-delta Ct method, in which delta Ct is calculated between gene of interest (GOI) and an average of reference genes (HKG), followed by delta-delta Ct calculations [delta Ct (Test Group)-delta Ct (Control Group)]. Fold Change is then calculated using 2^ (-delta delta Ct) formula. The data analysis web portal also plots scatter plot, volcano plot, clustergram, and heat map. This data analysis report was exported from the QIAGEN web portal at GeneGlobe.

Table 3. Gene table according RT² Profiler PCR Array (QIAGEN, Cat. no. PAHS-038Z)

Position	RefSeq Number	Symbol	Description
A01	NM_005502	ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1
A02	NM_000789	ACE	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1
A03	NM_000039	APOA1	Apolipoprotein A-I
A04	NM_000384	APOB	Apolipoprotein B (including Ag(x) antigen)
A05	NM_000041	APOE	Apolipoprotein E
A06	NM_004324	BAX	BCL2-associated X protein
A07	NM_000633	BCL2	B-cell CLL/lymphoma 2
A08	NM_004049	BCL2A1	BCL2-related protein A1
A09	NM_138578	BCL2L1	BCL2-like 1
A10	NM_001196	BID	BH3 interacting domain death agonist
A11	NM_001165	BIRC3	Baculoviral IAP repeat containing 3
A12	NM_002982	CCL2	Chemokine (C-C motif) ligand 2

B01	NM_002985	CCL5	Chemokine (C-C motif) ligand 5
B02	NM_001295	CCR1	Chemokine (C-C motif) receptor 1
B03	NM_001123396	CCR2	Chemokine (C-C motif) receptor 2
B04	NM_000610	CD44	CD44 molecule (Indian blood group)
B05	NM_001795	CDH5	Cadherin 5, type 2 (vascular endothelium)
B06	NM_003879	CFLAR	CASP8 and FADD-like apoptosis regulator
B07	NM_000090	COL3A1	Collagen, type III, alpha 1
B08	NM_000757	CSF1	Colony stimulating factor 1 (macrophage)
B09	NM_000758	CSF2	Colony stimulating factor 2 (granulocyte-macrophage)
B10	NM_001901	CCN2	Cellular communication network factor 2
B11	NM_001964	EGR1	Early growth response 1
B12	NM_000501	ELN	Elastin
C01	NM_000118	ENG	Endoglin
C02	NM_004102	FABP3	Fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)
C03	NM_000043	FAS	Fas (TNF receptor superfamily, member 6)
C04	NM_000508	FGA	Fibrinogen alpha chain
C05	NM_002006	FGF2	Fibroblast growth factor 2 (basic)
C06	NM_002026	FN1	Fibronectin 1
C07	NM_001945	HBEGF	Heparin-binding EGF-like growth factor
C08	NM_000201	ICAM1	Intercellular adhesion molecule 1
C09	NM_000874	IFNAR2	Interferon (alpha, beta and omega) receptor 2
C10	NM_000619	IFNG	Interferon, gamma
C11	NM_000575	IL1A	Interleukin 1, alpha
C12	NM_000877	IL1R1	Interleukin 1 receptor, type I
D01	NM_004633	IL1R2	Interleukin 1 receptor, type II
D02	NM_000586	IL2	Interleukin 2
D03	NM_000588	IL3	Interleukin 3 (colony-stimulating factor, multiple)
D04	NM_000589	IL4	Interleukin 4

D05	NM_000879	IL5	Interleukin 5 (colony-stimulating factor, eosinophil)
D06	NM_002203	ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)
D07	NM_002205	ITGA5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)
D08	NM_000887	ITGAX	Integrin, alpha X (complement component 3 receptor 4 subunit)
D09	NM_000211	ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)
D10	NM_002253	KDR	Kinase insert domain receptor (a type III receptor tyrosine kinase)
D11	NM_016270	KLF2	Kruppel-like factor 2 (lung)
D12	NM_005559	LAMA1	Laminin, alpha 1
E01	NM_000527	LDLR	Low density lipoprotein receptor
E02	NM_002309	LIF	Leukemia inhibitory factor (cholinergic differentiation factor)
E03	NM_005577	LPA	Lipoprotein, Lp(a)
E04	NM_000237	LPL	Lipoprotein lipase
E05	NM_002421	MMP1	Matrix metallopeptidase 1 (interstitial collagenase)
E06	NM_002422	MMP3	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)
E07	NM_002445	MSR1	Macrophage scavenger receptor 1
E08	NM_003998	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
E09	NM_000603	NOS3	Nitric oxide synthase 3 (endothelial cell)
E10	NM_000905	NPY	Neuropeptide Y
E11	NM_005693	NR1H3	Nuclear receptor subfamily 1, group H, member 3
E12	NM_002607	PDGFA	Platelet-derived growth factor alpha polypeptide
F01	NM_002608	PDGFB	Platelet-derived growth factor beta polypeptide
F02	NM_002609	PDGFRB	Platelet-derived growth factor receptor, beta polypeptide
F03	NM_001122	PLIN2	Perilipin 2

F04	NM_005036	PPARA	Peroxisome proliferator-activated receptor alpha
F05	NM_006238	PPARD	Peroxisome proliferator-activated receptor delta
F06	NM_015869	PPARG	Peroxisome proliferator-activated receptor gamma
F07	NM_000962	PTGS1	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)
F08	NM_002957	RXRA	Retinoid X receptor, alpha
F09	NM_000450	SELE	Selectin E
F10	NM_000655	SELL	Selectin L
F11	NM_003006	SELPLG	Selectin P ligand
F12	NM_002575	SERPINB2	Serpin peptidase inhibitor, clade B (ovalbumin), member 2
G01	NM_000602	SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
G02	NM_000454	SOD1	Superoxide dismutase 1, soluble
G03	NM_000582	SPP1	Secreted phosphoprotein 1
G04	NM_000660	TGFB1	Transforming growth factor, beta 1
G05	NM_003238	TGFB2	Transforming growth factor, beta 2
G06	NM_003248	THBS4	Thrombospondin 4
G07	NM_002160	TNC	Tenascin C
G08	NM_000594	TNF	Tumor necrosis factor
G09	NM_006290	TNFAIP3	Tumor necrosis factor, alpha-induced protein 3
G10	NM_001078	VCAM1	Vascular cell adhesion molecule 1
G11	NM_003376	VEGFA	Vascular endothelial growth factor A
G12	NM_000552	VWF	Von Willebrand factor
H01	NM_001101	ACTB	Actin, beta
H02	NM_004048	B2M	Beta-2-microglobulin
H03	NM_002046	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H04	NM_000194	HPRT1	Hypoxanthine phosphoribosyltransferase 1
H05	NM_001002	RPLP0	Ribosomal protein, large, P0
H06	SA_00105	HGDC	Human Genomic DNA Contamination

H07	SA_00104	RTC	Reverse Transcription Control
H08	SA_00104	RTC	Reverse Transcription Control
H09	SA_00104	RTC	Reverse Transcription Control
H10	SA_00103	PPC	Positive PCR Control
H11	SA_00103	PPC	Positive PCR Control
H12	SA_00103	PPC	Positive PCR Control

A pre-amplification using the appropriate species- and pathway-specific RT² PreAMP Primer Mix was not performed and no corrections were made to C_t values during the data analysis procedure other than the use of the C_t cut-off value. The C_t cut-off was set to 35. Fold regulation and p-value cut-off were set to 2 and 0.05, respectively.

To assess gene expression we compared the following groups of samples:

1. **Normal HCMV POSITIVE** placenta (test group) to **Normal HCMV NEGATIVE** placenta (control group)
2. **POSITIVE HCMV PE** placenta (test group) to **NEGATIVE HCMV PE** placenta (control group)
3. **POSITIVE HCMV PE** placenta (test group) to **Normal POSITIVE HCMV** placenta (control group)

Histopathological analysis

Frozen sections of 5 µm from placental biopsies were done using a cryotome. They were fixed in cold pure acetone solution (90%) for 20 minutes. Haematoxylin-eosin-saffron (HES) staining was automatically done in order to look for the detachment of the arterial intima. The stained sections were analyzed with a digital slide scanner, Hamamatsu® NanoZoomer and the NDP viewer software.

Immunostaining was also performed in order to look for HCMV presence within placental blood vessels. Multiple markers were targeted with an indirect immunofluorescence protocole. The targets were endothelial cells (CD31), smooth muscle cells (alpha actin smooth muscle), macrophages (CD 68), HCMV IE antigens and DAPI. The antibodies used for immunofluorescence technique and the dilutions are shown in table 4.

Table 4. Antibodies and dilutions used for immunostaining

Target	Unconjugated antibodies	Dilution	Conjugated antibodies	Dilution
EC	-	-	**Ozyme SigmaAldrich** BLE303126, Alexa Fluor 594 anti-human CD31	1/50
Macrophages	-	-	**Abcam** Recombinant Alexa Fluor® 647 Anti-CD68 antibody [EPR20545] (ab224029)	1/50
SMC	-	-	**Termo-Fischer** 25UG AntiAlpha-Smooth Muscle Actin eFluor 570	1/1500
HCMV IE antigen	**Abcam** Anti-CMV IE1 and IE2 antibody [CH160] (ab53495)	1/50	**Abcam** Goat Anti-Mouse IgG Alexa Fluor® 488 (ab150113)	1/200
Nucleus	Abcam DAPI Staining Solution (ab228549)	1/1000	-	

Fixed frozen sections were washed out with PBS solution 3 times for 5 minutes. Further more cell permeabilization was done with PBS-Triton 0.1% for 5 minutes at room temperature. Another wash out was performed with PBS 3 times for 5 minutes. Then, non-specific sites were fixed with PBS-BSA 0.5% for 30 minutes at room temperature. Further, 50 µl of unconjugated mouse anti HCMV IE antigens antibodies (dilution 1/50) were added to sections followed by overnight incubation at 4 degrees. The next day a washout was done using a stirring platform – 3 times for 10 minutes. Next, 1500 µl mix of conjugated anti CD31, anti CD68, anti SMC and anti HCMV IEA (goat anti mouse) was obtained, respecting the above mentioned dilutions for each specific antibody. This mix

was then added to sections and incubated for one hour at 37 degrees. After this, a new washout -3 times x 10 minutes was done. The next step was DAPI staining with 1/1000 DAPI solution dilution for 5 minutes in a dark place, at room temperature. A last washout – 3 times x5 minutes was performed using PBS solution. In the end the sections were fixed with special liquid and varnished. Before confocal microscope analysis all sections were stored away from light at 4 degrees. For analysis a confocal Carl Zeiss Zen2 mycroscope was used. The image aquisition and processing was performed with ImageJ software, at the confocal microscopy platform (Biscem Inserm platform, Limoges University).

5.3 Results and discussions

Clinical data and risk factors analysis in relation to preeclampsia

For the test group, out of 52 patients included in this study, 33 (63%) had latent HCMV infection. The patients characteristics are presented in table 5. There were no statistically significant differences between the HCMV positive PE women and the negative ones in terms of early onset of severe preeclampsia incidence, nor in the presence of risk factors like in vitro fertilization, hypertension, presence of gestational diabetes, BMI >25kg/m2, smoking. Only multiple pregnancies were statistically significant in HCMV positive group compared to negative one (p value 0.001).

Table 5. Study group characteristics

Variable **N, (%)**	**Total** **N=52**	**HCMV+** **N= 33**	**CMV-** **N=19**	**p value**
Mean age	33.15 ±5.3	33.06±6.73	33.31±8.61	0.88
Smoking	14 (26.92%)	9 (27.27%)	5 (26.31%)	0.96
Preexistent hypertension	5 (9.61%)	4 (12.12%)	1 (5.26%)	0.68
Pregnancy diabetes	8 (15.38%)	5 (15.15%)	3 (15.78%)	0.97
In vitro fertilization	8 (15.38%)	3 (9.09%)	5 (26.31%)	0.31
BMI> 25 kg/m2	26 (50%)	15 (45.45%)	11 (57.89%)	0.13
Multiple pregnancies	30 (57.69%)	25 (75.75%)	5 (26.31%)	0.001

Early-onset PE	31 (59.61)	19 (57.57%)	12 (63.15%)	0.58
Severe PE	21 (40.38)	14 (42.42%)	8 (42.10%)	0.96

For the control group 10 patients were HCMV positive (50%) and 10 HCMV negative. No statistically differences were identified in terms of pregnancy and cardiovascular risk factors (the same as in the study group). To note that the control group was designed for genetic tests rather than for seroprevalence or clinical and risk factors analysis. The samples for the control group were not randomly selected. We chose to have 10 HCMV positive and 10 HCMV negative normal placentas to compare the gene expression in the three groups previously described.

In our study group the severity of preeclampsia was directly influenced by the simultaneous action of body mass index greater than 25 kg /m2 and positive IgG anti-HCMV antibodies serostatus (p-value 0.005), figure 11.

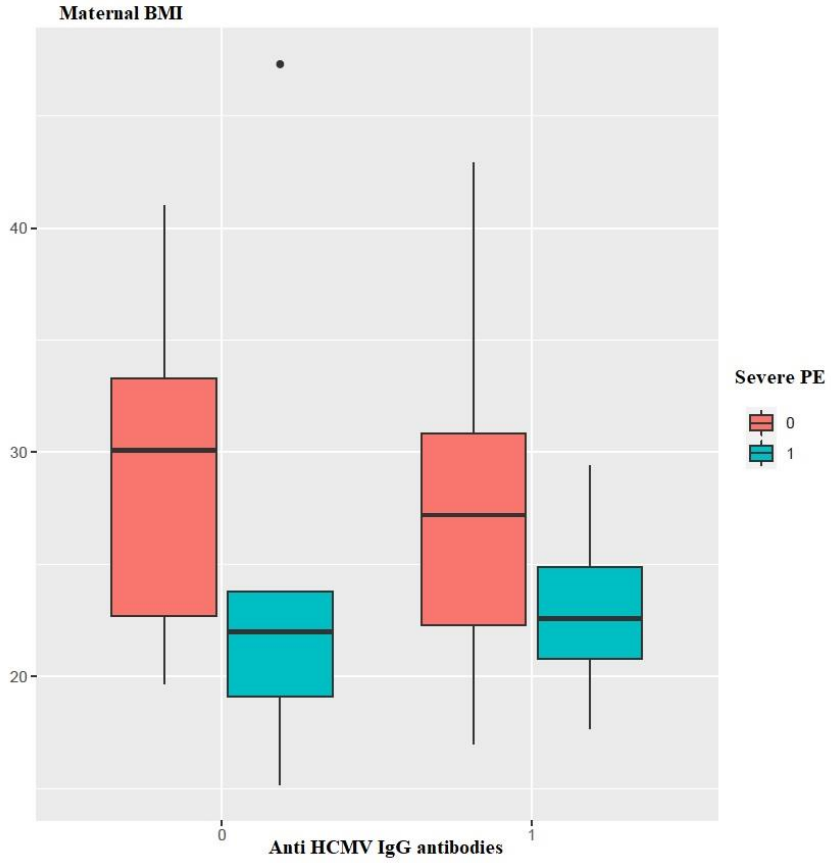

Figure 11. Maternal HCMV- positive serostatus and BMI greater than 25 kg/m2 influence the onset of severe PE

HCMV latent infection in association with advanced maternal age equally influenced the severity of PE (p-value 0.03), figure 12.

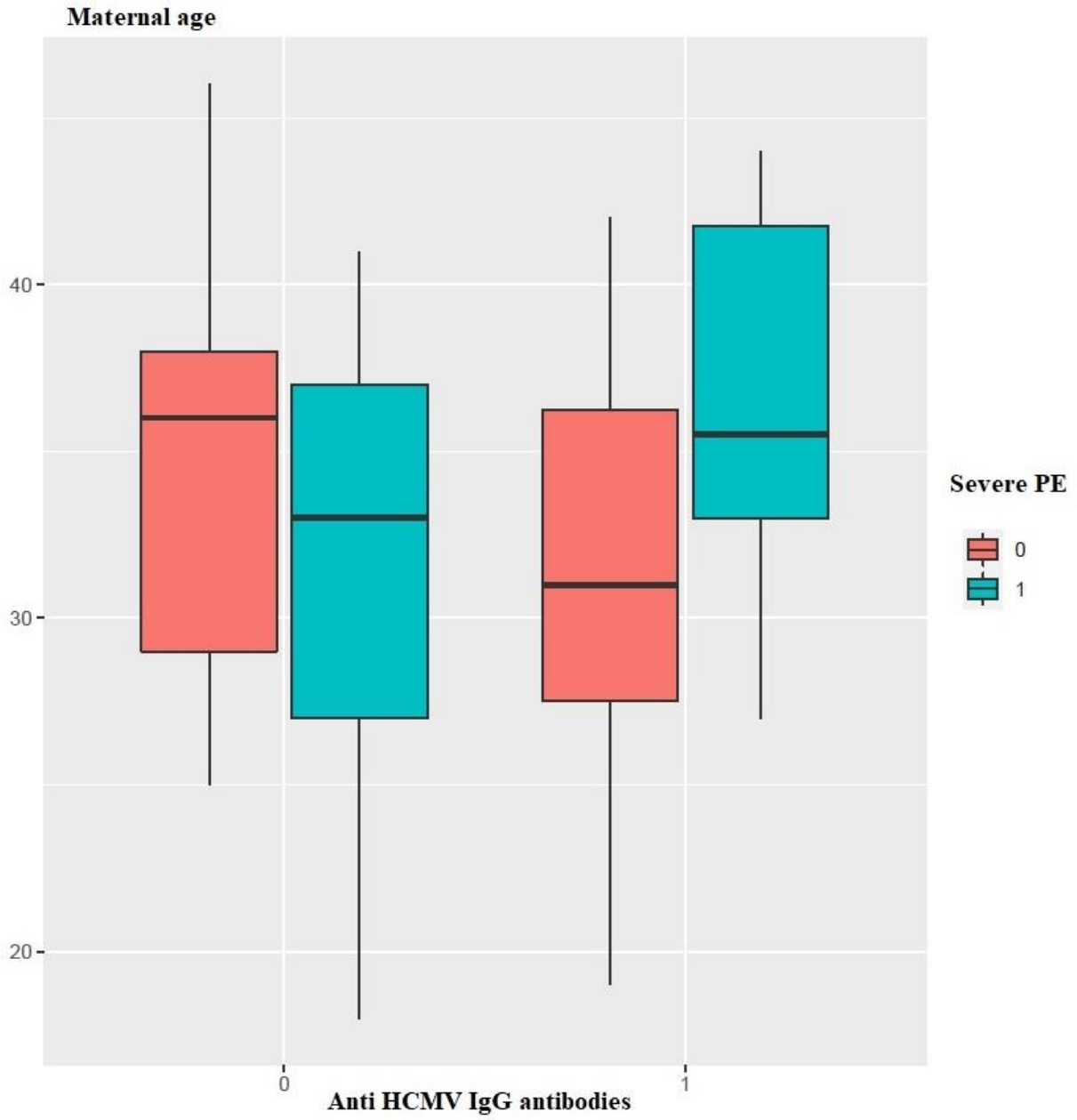

Figure 12. Simultaneous action of advanced maternal age and HCMV seropositive status influence the development of severe preeclampsia

As expected the early onset of preeclampsia and its severity was associated to low birth weight. However, our data suggest that birth weight in severe preeclampsia is highly influenced by positive HCMV IgG serostatus (p-value 0.0004), figure 13.

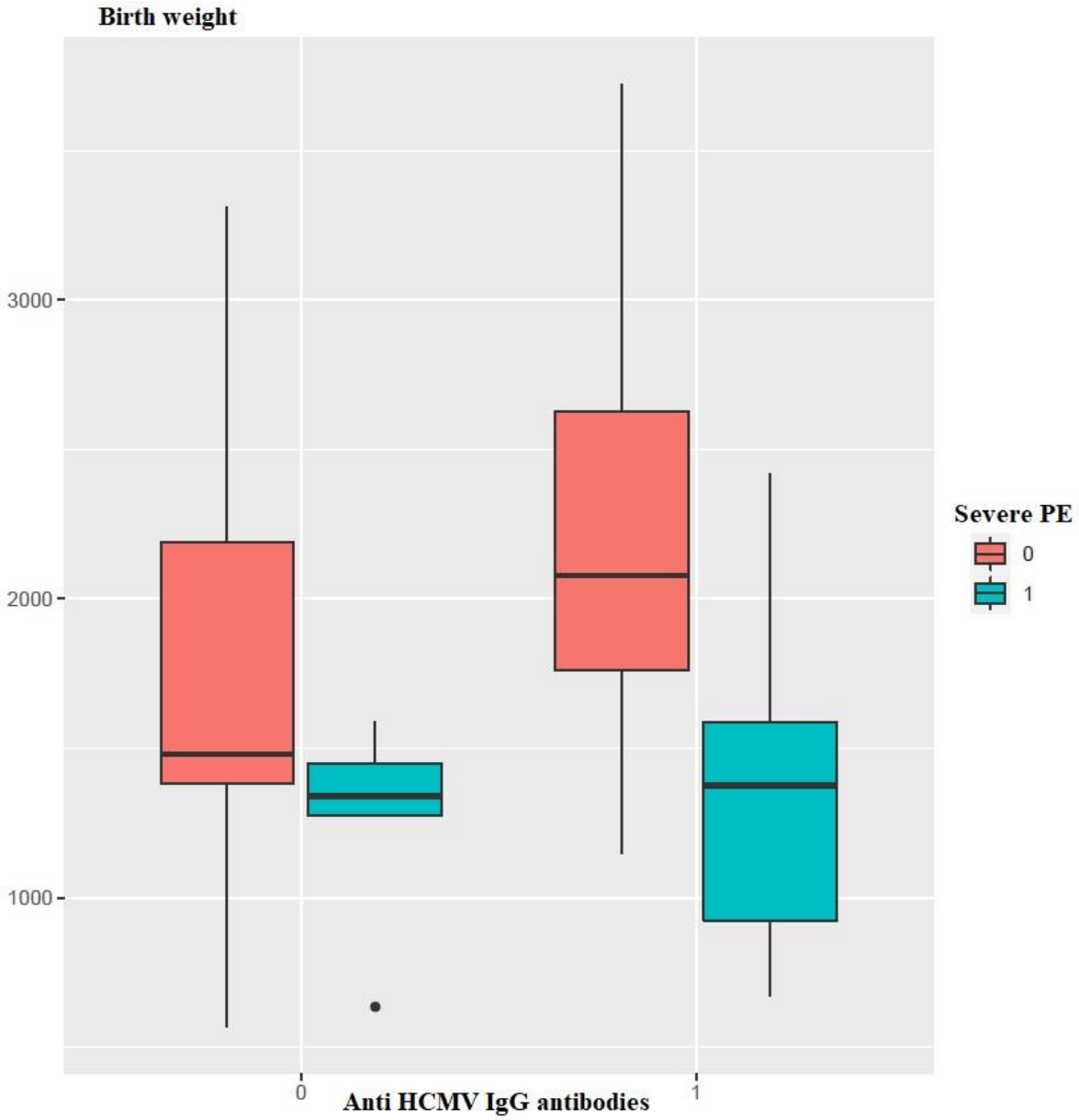

Figure 13. Low birth weight is highly influenced by positive HCMV IgG serostatus in severe PE

Genetic tests analysis

The information about RNA quality and quantity for the 12 placental biopsy samples used in this part of the study is shown in Table 6.

	Sample Name	Species	Source	Concentration (ng/ul）	Vol (µl)	Note	Total Amount (ng)	RIN	OD 260/280	OD 260/230
Sample Information*	P3658	Human	Placenta	393,50	10	Normal pregnancy HCMV negative	3935	5,4	2.06	2.07
	P3594	Human	Placenta	281,46	10	Normal pregnancy HCMV negative	2814,6	6,5	2.12	1.91
	P3987	Human	Placenta	398,52	10	Normal pregnancy HCMV negative	3985,2	6,2	2.07	1.70
	P3618	Human	Placenta	645,87	10	Normal pregnancy HCMV positive	6458,7	5,8	2.08	2.10
	P3968	Human	Placenta	530,64	10	Normal pregnancy HCMV positive	5306,4	5,7	2.08	2.09
	P3669	Human	Placenta	433,17	10	Normal pregnancy HCMV positive	4331,7	6,4	2.04	1.71
	P2792	Human	Placenta	286,94	10	Preeclampsia HCMV negative	2869,4	5,5	2.06	1.99
	P3043	Human	Placenta	115,66	10	Preeclampsia HCMV negative	1156,6	5,4	2.09	1.37
	P3508	Human	Placenta	336,20	10	Preeclampsia HCMV negative	3362	4,9	2.01	1.76
	P2334	Human	Placenta	239,70	10	Preeclampsia HCMV positive	2397	5,1	2.07	1.82
	P2796	Human	Placenta	589,95	10	Preeclampsia HCMV positive IgM+, IgG+	5899,5	5,7	2.11	2.08
	P2803J2	Human	Placenta	418,71	10	Preeclampsia CMV positive, IgM eqv, IgG+	4187,1	5,2	2.03	2.00

Table 6. The quantity and quality of RNA used for genetic testing

1. **Normal HCMV POSITIVE** placenta (test group) vs **Normal HCMV NEGATIVE** placenta (control group)

For this group we identified 5 upregulated genes and two down-regulated genes in the test group when considering only the fold regulation cut-off (figure 14). Except for APOA1 that had a low expression in both groups, with a Ct higher than 30, all other genes were normally or highly expressed in both groups.

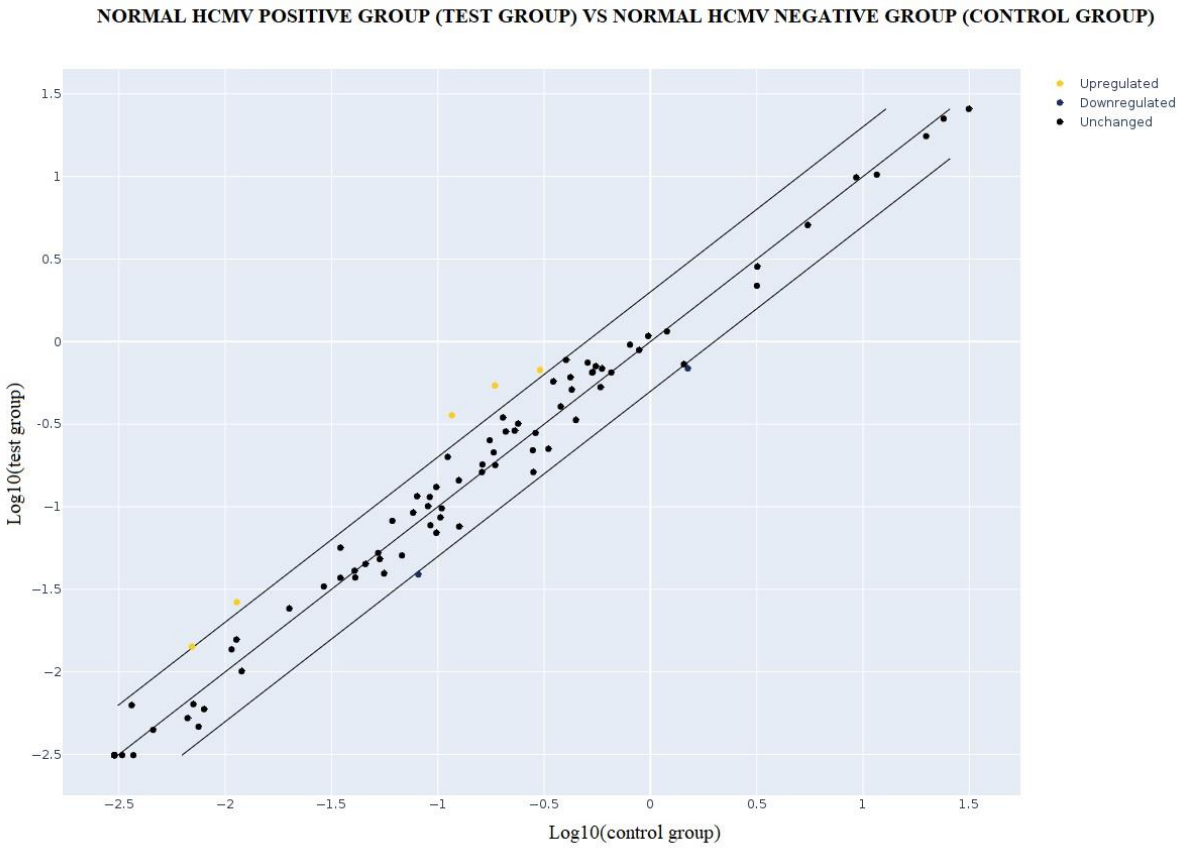

Figure 14. Gene expression assessed by fold regulation cut-off in NORMAL HCMV POSITIVE group vs NORMAL HCVM NEGATIVE group

However, statistically, only 2 gene expression were relevant for this test group as seen in table 7.

Table 7. Fold regulation and p-value for genes over-expressed and under-expressed in the test group

Genes over-expressed in TEST GROUP vs CONTROL GROUP

Position	Gene symbol	Fold regulation	p - Value	RT^2 qPCR Assay Catalog #
A03	APOA1	2.04	0.369293	PPH02633B
A07	BCL2	3.08	0.145401	PPH00079B
D08	ITGAX	2.33	0.047123	PPH00661F
E04	LPL	2.92	0.208621	PPH00023C
G04	TGFB1	2.22	0.246380	PPH00508A
Genes under-expressed in TEST GROUP vs CONTROL GROUP				
Position	**Gene symbol**	**Fold regulation**	**p - Value**	**RT^2 qPCR Assay Catalog #**
B11	EGR1	-2.08	0.285372	PPH00139A
G10	VCAM1	-2.18	0.048488	PPH00623E

For better visualization of biological and statistical gene expression in the test group vs control group a volcano plot was performed, figure 15.

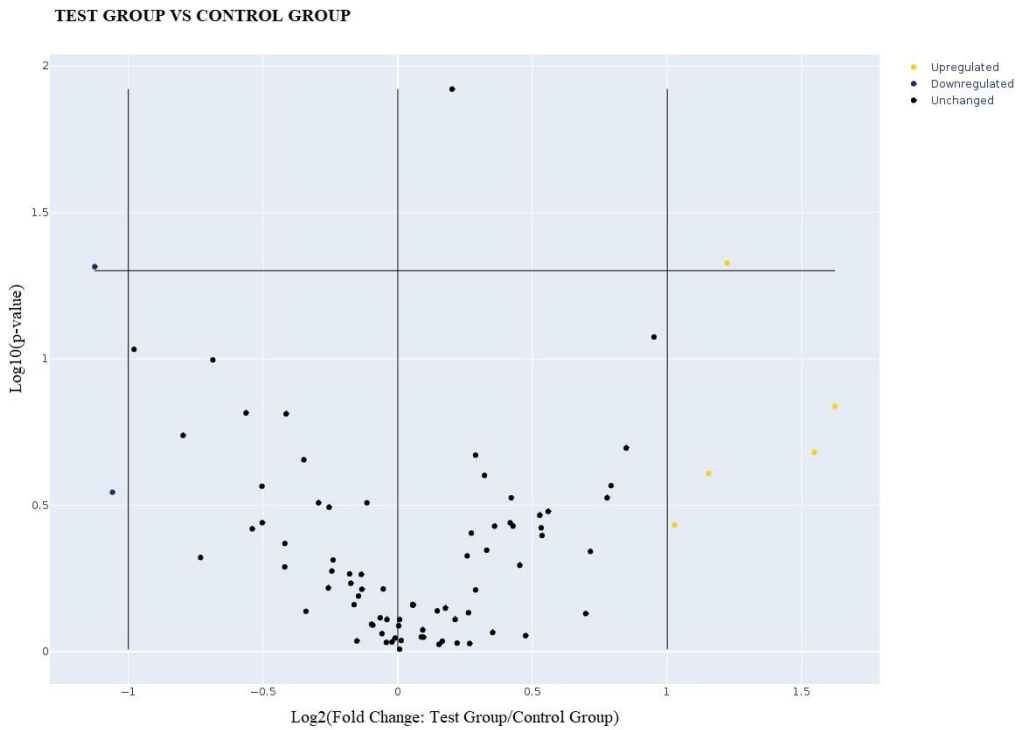

Figure 15. Volcano plot - Biological and statistical relevant gene expression in Normal HCMV positive placentas vs Normal HCMV negative placentas.

VCAM-1 gene encodes for vascular adhesion molecule which was identified within aortic atherosclerotic plaques in human [114]. It may not be expressed in normal conditions, but it seems to increase significantly in pro-atherogenic conditions like inflammation or shear stress [93]. Studies conducted in pregnant women have shown low soluble VCAM-1 expression in normal pregnancies, but however increased levels were measured in mild and severe preeclampsia [50,88,124]. The current data offer a controversial opinion on VCAM-1 expression in HCMV infection. Some in vitro studies conclude there is no change in VCAM-1 expression during HCMV infection [140] while others identify over-expression of this gene in HCMV infected ECs [171]. An in vivo study suggest VCAM-1 increased expression in latent HCMV infection in pregnancy [11]. However, in our study, VCAM-1 expression is downregulated in normal HCMV positive placentas when compared to normal HCMV negative placentas.

2. **POSITIVE HCMV PE** placenta (test group) vs **NEGATIVE HCMV PE** placenta (control group)

No statistically significant results were obtained for this group (table 8). However, biologically, there were some over-expressed and under-expressed genes in the test group compared to control, figure 16. All these genes were low expressed, Ct>30 in both groups, which makes the results difficult to interpret.

Table 8. Fold regulation and p-value for genes over-expressed and under-expressed in the PE HCMV positive group when compared to PE HCMV negative group

Genes over-expressed in TEST GROUP vs CONTROL GROUP				
Position	**Gene symbol**	**Fold regulation**	**p - Value**	**RT^2 qPCR Assay Catalog #**
C04	FGA	2.07	0.310316	PPH02623E
Genes under-expressed in TEST GROUP vs CONTROL GROUP				
Position	**Gene symbol**	**Fold regulation**	**p - Value**	**RT^2 qPCR Assay Catalog #**
C02	ENG	-2.15	0.235923	PPH01140G
D05	IL5	-2.57	0.187076	PPH00692B
E04	LPL	-2.33	0.206788	PPH00023C
F03	PLIN2	-2.26	0.363180	PPH02583A

These results may suggest that HCMV infection alone does not influence the evolution of PE so much, but it may also suggest more important vascular injury in PE HCMV positive group as a result of FGA over-expression that encodes for fibrinogen alpha chain fraction.

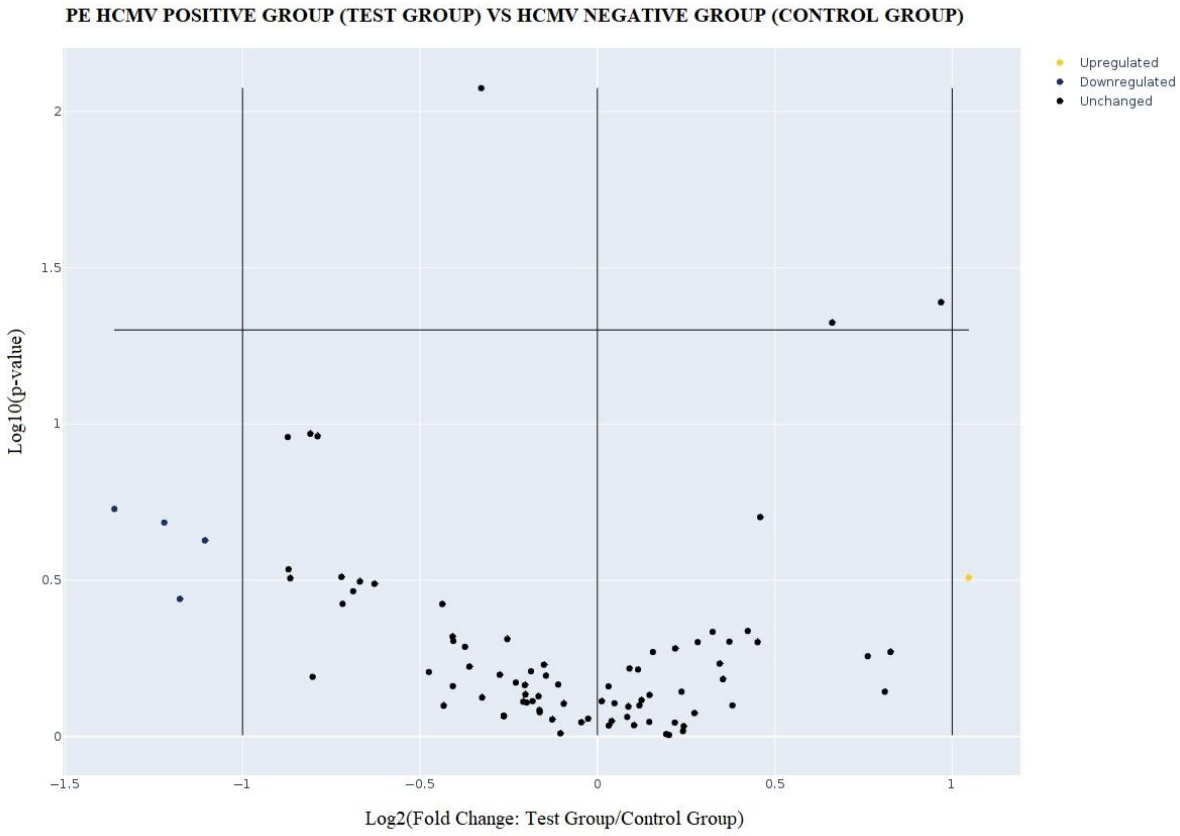

Figure 16. Volcano-plot - Biological and statistical overview of over-expressed and under-expressed genes in PE HCVM positive placentas vs PE HCMV negative placentas

1. **POSITIVE HCMV PE** placenta (test group) vs **Normal POSITIVE HCMV** placenta (control group)

For this group we have noticed important differences in gene expression (table 9). How much of these differences are influenced by PE or HCMV alone or in association is a question to be answered in further studies.

Table 9. Fold regulation and p-value for genes over-expressed and under-expressed in the PE HCMV positive group when compared to Normal HCMV positive group

Genes over-expressed in TEST GROUP vs CONTROL GROUP				
Position	**Gene symbol**	**Fold regulation**	**p - Value**	**RT² qPCR Assay Catalog #**
B11	EGR1	2.63	0.115389	PPH00139A
C04	FGA	2.50	0.129665	PPH02623E
C06	FN1	4.45	0.084705	PPH00143B
D01	IL1R2	2.18	0.252537	PPH00313C
D03	IL3	2.22	0.162121	PPH00691B
D12	LAMA1	3.06	0.030257	PPH02584A
E02	LIF	2.35	0.141016	PPH00813F
E06	MMP3	2.79	0.067224	PPH00235F
G01	SERPINE1	4.24	0.084253	PPH00215F
G05	TGFB2	2.52	0.089603	PPH00524B
G09	TNFAIP3	2.79	0.109264	PPH00063A
Genes under-expressed in TEST GROUP vs CONTROL GROUP				
Position	**Gene symbol**	**Fold regulation**	**p - Value**	**RT² qPCR Assay Catalog #**
A07	BCL2	-4.59	0.106776	PPH00079B
D09	ITGB2	-2.51	0.025340	PPH00679F
E04	LPL	-4.80	0.161255	PPH00023C
E05	MMP1	-4.69	0.055702	PPH00120B
F10	SELL	-2.08	0.047707	PPH00677F
F12	SERPINB2	-3.19	0.219184	PPH00793C
G03	SPP1	-2.28	0.037309	PPH00582E

ERG1, FGA, IL1R2, IL3, LIF, MMP3, MMP1 had a low expression in both test and control group. The magnitude of gene expression in each sample tested in this group is presented in figure 17. Although not all the above-mentioned genes were statistically significant, figure 18, it is worth mentioning that most of the over-expressed genes in the PE HCMV positive group have a pro-inflammatory role and were often associated with atherosclerosis. Thus, IL1R2 gene encodes for IL1 cytokine receptor type 2 that plays an important role in cell metabolism and in immune response regulation and it was often associated to atherosclerosis and acute myocardial infarction [128,170]. ERG1 is highly expressed in animal model atherosclerosis. It modulates the activity of genes like PDGF and TGF-β and its over-expression may trigger vascular injury [102]. LAMA1 encodes for extracellular adhesion molecules and was also associated to cell recruitment in atherosclerosis [9].

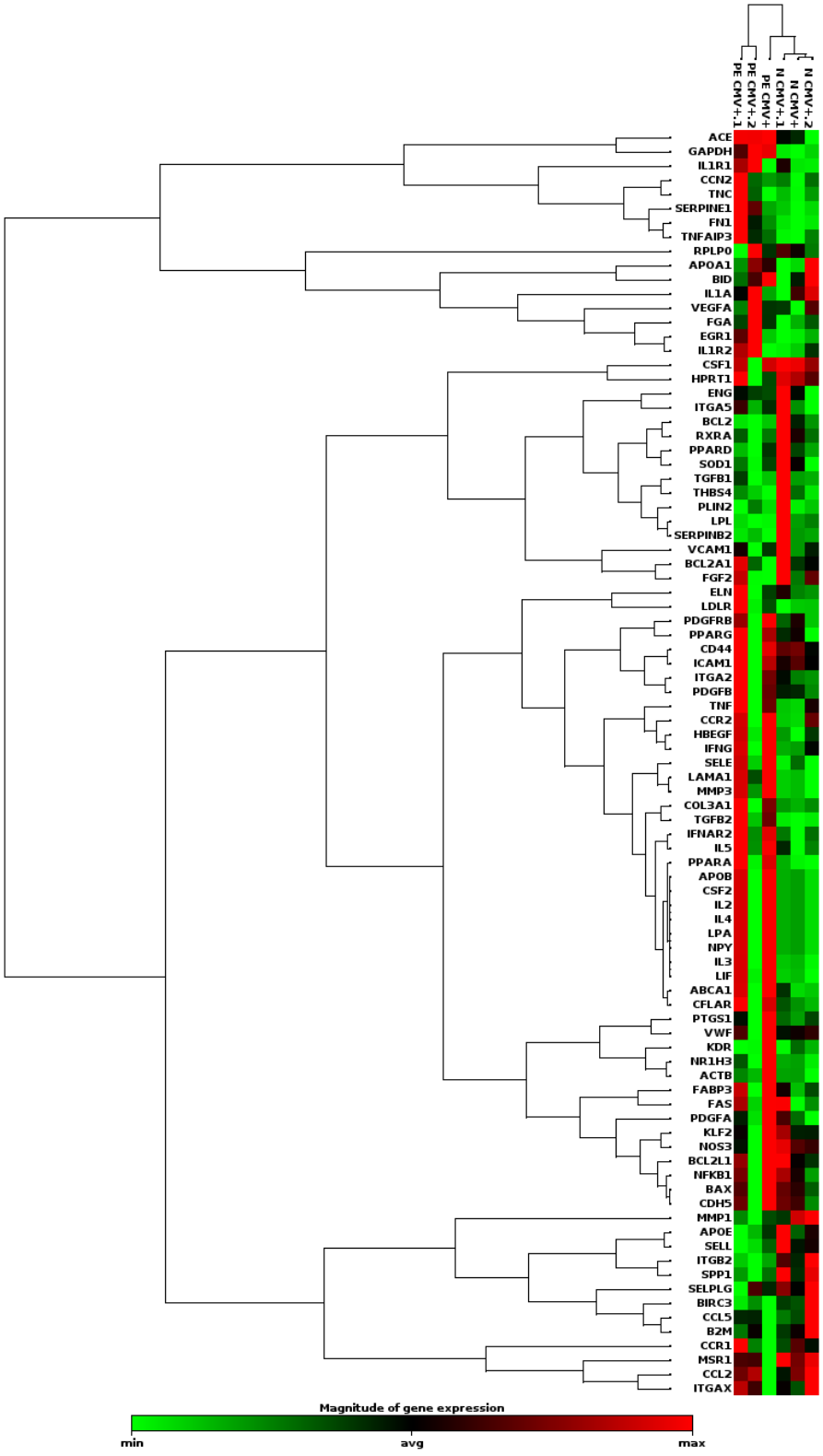

Figure 17. Clustergram of gene expression in PE HCMV positive group vs Normal HCMV positive group

In contrast to most literature findings, in our last group important pro-atherogenic genes encoding for adhesion molecules or proteins involved in angiogenesis, immune

modulation, leukocytes trafficking like ITGB2, SELL and SPP1 [22,37,48] are biologically and statistically relevant down-regulated, figure 18.

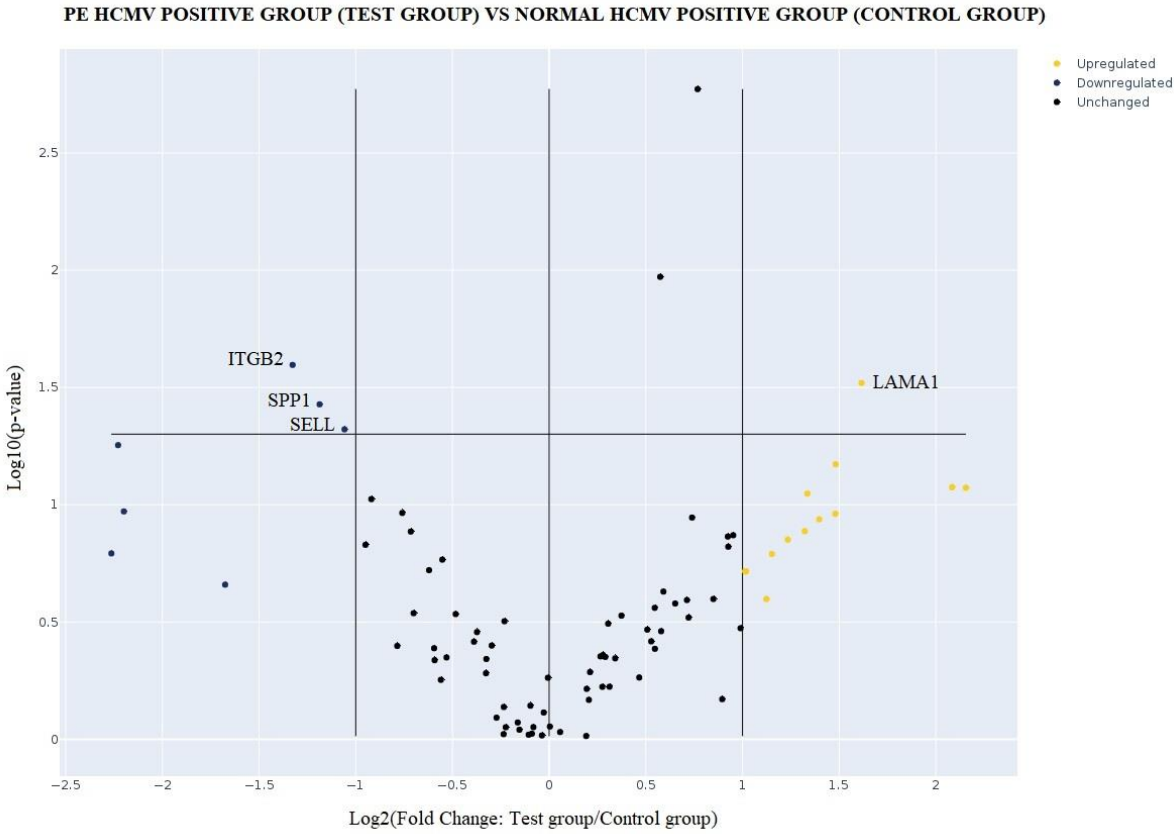

Figure 18. Volcano-plot - Biological and statistical overview of over-expressed and under-expressed genes in PE HCVM positive placentas vs NORMAL HCMV positive placentas

Although clinical data suggest an important influence of HCMV infection in preeclampsia, the gene expression results are not so conclusive. The heterogeneity of gene expression in our groups may be due to some important biases of this work:

1. unequal distribution of HMCV within the placenta which keeps an increased hazard ratio as we used placental biopsies for our genetic tests and not the whole placenta
2. the low number of samples (3 per group) used for genetic tests compared to the 52 medical records used to assess the clinical outcomes
3. the integrity, “full wall” placental biopsy; it is well known that acute atherosis or vascular remodeling in pregnancy targets especially spiral arteries that are found on the maternal face of the placenta and are much more difficult to obtain comparing to chorionic villi arteries. Furthermore, spiral arteries are also different in structure

as they have a better developed arterial wall, especially the muscular layer, compared to chorionic villi arteries.

Histopathological findings

A total of 1500 sections were analyzed for HES staining and 50 sections for immunostaining. We searched for acute atherosis lesions as well as for any signs of endothelial injury, SMCs migration within the intima or HCMV antigen presence. 278 HES sections were excluded due to architectural disorganization (figure 19) secondary to impairment in freezing process or frozen sections handling. All 50 immunostained sections were in good condition and were fully analyzed.

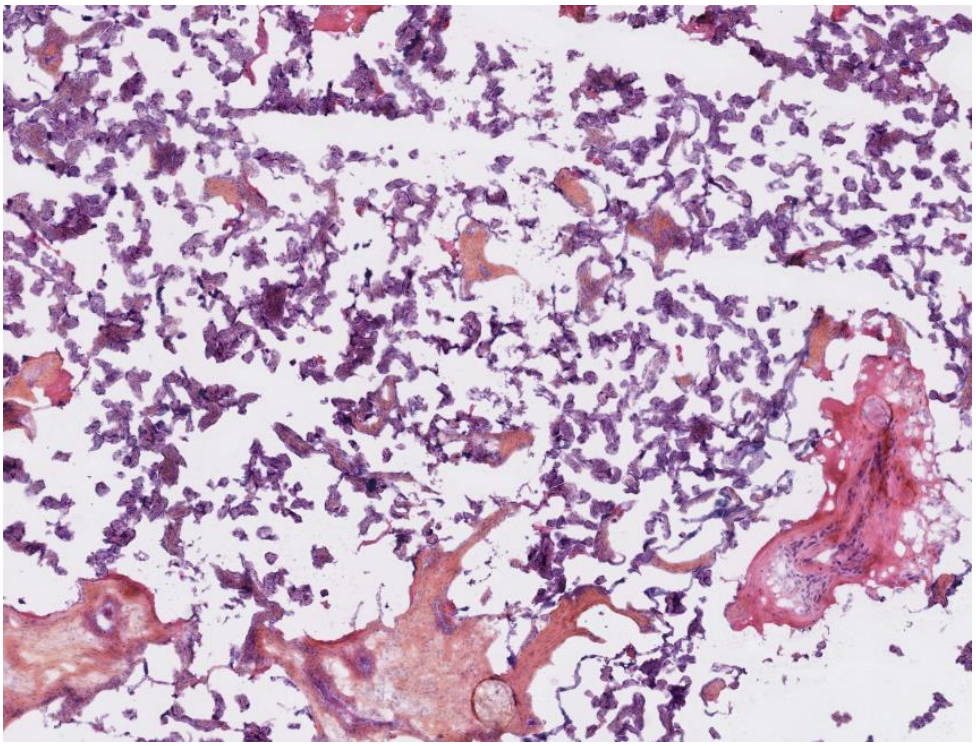

Figure 19. Architectural disorganization due to frozen section technique impairment in normal HCMV negative placental biopsy

Not only that there are not so many studies in literature with histopathological details of placental vascularization, but almost all of them are using paraffin embedded tissue. This was one of the reasons that the whole histology protocol took us almost two years to obtain good sections to analyze. Few possible acute atherosis lesions were identified after analyzing all HES sections, figure 20.

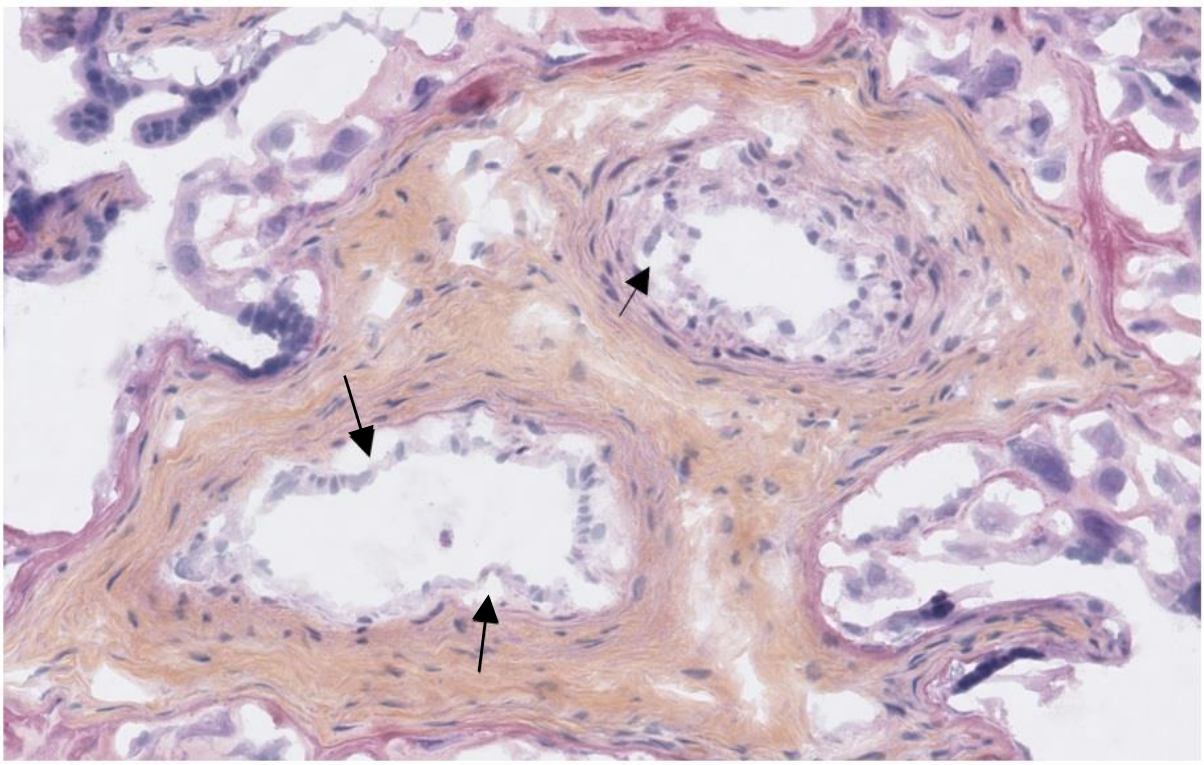

Figure 20. Intimal detachment (black arrow) in mild PE in positive anti HCMV IgG obese (BMI 44 kg/m2) patient (HES staining)

The analysis of immunofluorescent stained sections did not return significant results, unfortunately. All our sections captured images of arteries from the chorionic villi, figure 21. There was no HCMV antigen presence within the chorionic villi arterial wall, nor any SMC or macrophages present within the intimal layer.

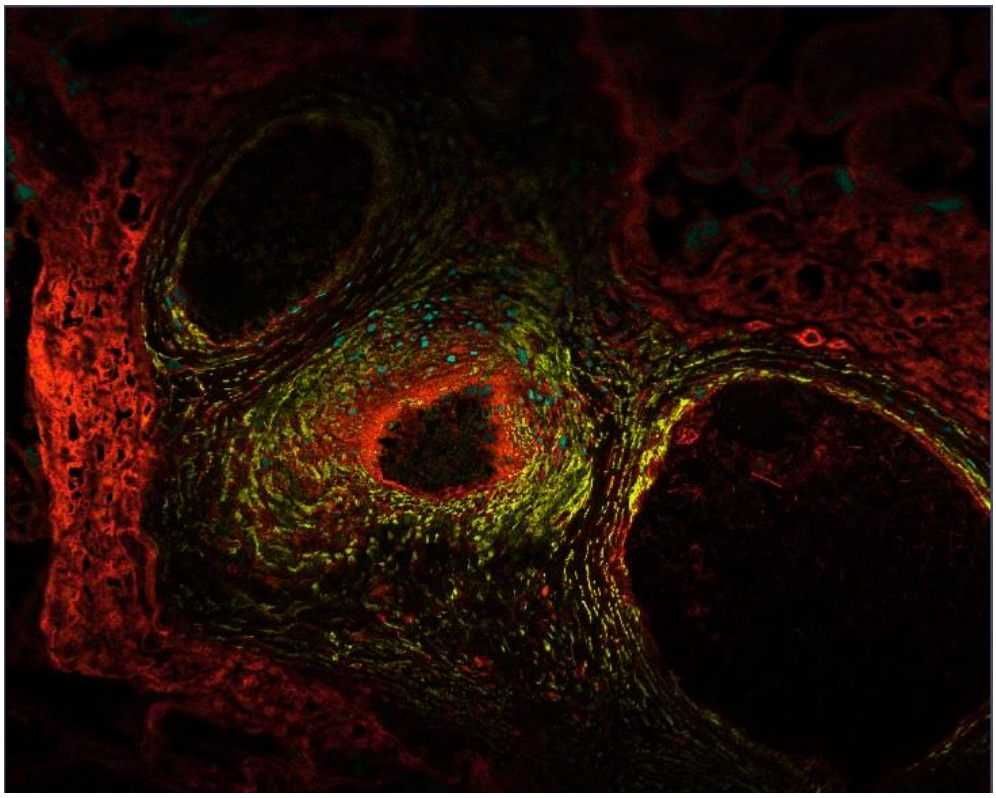

Figure 21. Immunostaining of chorionic villi vascularization. An artery in the middle with 2 lateral veins. Red – Ecs, Yellow SMCs, Light Blue - DAPI

One important aspect for our histopathological findings is using frozen biopsies retrospectively. The tubes dimensions and tissue folding before freezing were the two major problems in sectioning and correct orientation of the biological sample. Although there were full placental wall biopsies it was hard to obtain a complete image with the maternal and fetal surface on the same section. However, we had the opportunity to deepen our knowledge on chorionic villi vascularization and to find possible acute atherosis lesions at this level, not only in spiral arteries as previously described.

Placentas are “hard to get” biological specimens due to many regulations in this regard. To our knowledge this is one of the largest cohorts of placental specimens that were studied until now. Therefore, although the gene expression results are not very clear, we succeeded in establishing the work protocols that will help us to develop our further projects on this topic. However, the clinical results and HES histopathological findings may be considered enough arguments to show that HCMV infection influences vascular remodeling and PE outcomes.

CHAPTER 6. DEEP SEQUENCING ANALYSIS OF PLACENTAL TRANSCRIPTOMICS

6.1 Background

As our first results were not very clear, we studied the transcriptomics from the same 12 placentas selected for the previous work to have a broader view of the potential transcriptomic modification together with HCMV specific transcripts signature. To our knowledge no other similar study on placenta was done before.

6.2 Materials and methods

RNA from placental biopsies were selected according to their quantity and quality. We used the same samples as described in table 6. The total RNA samples were sent in dry ice to Novogene (UK) for transcriptomic analysis. Transcriptomics were performed by RNA seq Illumina and bioinformatic analyses after validation of run quality were conducted to 1) search for HCMV transcripts and 2) analyse the discrepancies of expression of genes and their involvement in known pathways. Samples were grouped for analysis as indicated below, in table 10, in 5 groups A1, A2, A3, A4, and A5. The latter, A5, represents all the preeclampsia placentae, whatever the HCMV status.

Table 10. Placental specimen group distribution for transcriptomics analysis

P3658	Placenta	Normal pregnancy HCMV negative	A1	
P3594	Placenta	Normal pregnancy HCMV negative	A1	
P3987	Placenta	Normal pregnancy HCMV negative	A1	
P3618	Placenta	Normal pregnancy HCMV positive	A2	
P3968	Placenta	Normal pregnancy HCMV positive	A2	
P3669	Placenta	Normal pregnancy HCMV positive	A2	

P2792	Placenta	PE HCMV negative	A3	A5
P3043	Placenta	PE HCMV negative	A3	A5
P3508	Placenta	PE HCMV negative	A3	A5
P2334	Placenta	PE HCMV positive	A4	A5
P2796	Placenta	PE HCMV positive for both IgM and IgG	A4	A5
P2803J2	Placenta	PE HCMV positive, IgM equivocal, IgG+	A4	A5

6.3 Library preparation and sequencing by Novogene

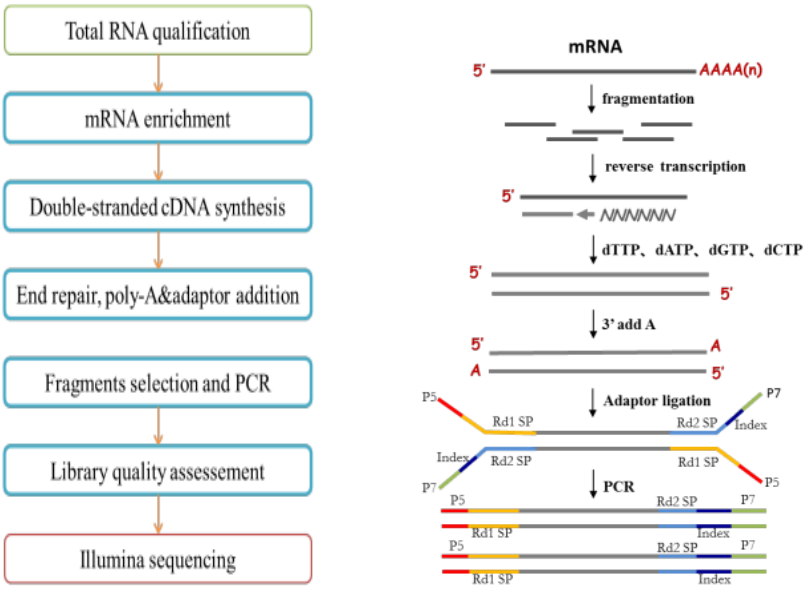

Figure 22. Novogene sequencing protocole

Raw data were trimmed, first aligned after substraction of hg19 human genome sequences against Merlin sequence for HCMV and in parallel aligned against hg19 human genome (figure 22). The groups were then compared to identify specific mutations or patterns (analysis performed both by Novogene and by Limoges genomic platform by the National Reference Center - bioinformatics engineer V. Tilloy).

6.4 Results and discussions

Raw sequences were of high quality, but no HCMV sequence could be detected at a significant level (table 11).

	nb reads total	nb reads hg19	%hg19	nb reads sans hg19	nb reads Merlin	%merlin
P2334_4	94206226	89911901	95,4	2085309	45	0,000048
P2792_3	94291286	88434353	93,8	3180028	71	0,000075
P2796_4	77594184	74980671	96,6	1074025	4	0,000005
P2803J24	100927458	94465261	93,6	3417914	39	0,000039
P3043_3	115081358	108494289	94,3	3526854	50	0,000043
P3508_3	94360072	91260042	96,7	1204837	2	0,000002
P3594_1	91703410	88062031	96,0	1532660	21	0,000023
P3618_2	90788376	86782793	95,6	1718479	16	0,000018
P3658_1	104370672	100483761	96,3	1551123	17	0,000016
P3669_2	75343246	71831971	95,3	1503684	13	0,000017
P3968_2	87969154	84906108	96,5	1186699	9	0,000010
P3987_1	90671504	87135668	96,1	1401779	22	0,000024

Table 11. Raw human and HCMV sequences

Run quality control showed excellent quality, figure 23, and very high quality of mapping against hg19 human genome after pair-end analysis. The reads quality was excellent in both reading directions, figure 24, with a very homogeneous quality of coverage for all samples, figure 25.

Sample	raw_reads	clean_reads	raw_data(G)	clean_data(G)	error_rate(%	Q20(%)	Q30(%)	GC_content(%)
P3987_1	46732991	45335752	14.0	13.6	0.04	97.72	93.78	49.61
P3043_3	58909837	57540679	17.7	17.3	0.04	97.48	93.30	49.08
P3594_1	47523252	45851705	14.3	13.8	0.04	97.28	92.79	49.27
P2792_3	48925393	47145643	14.7	14.1	0.04	97.27	92.81	49.72
P2796_4	39422112	38797092	11.8	11.6	0.04	97.66	93.63	49.87
P3658_1	52952982	52185336	15.9	15.7	0.04	97.60	93.49	48.84
P3669_2	38376292	37671623	11.5	11.3	0.04	97.82	94.01	49.50
P2803J24	51367073	50463729	15.4	15.1	0.04	96.96	92.13	48.55
P3618_2	46032219	45394188	13.8	13.6	0.04	97.44	93.11	49.28
P3968_2	44439547	43984577	13.3	13.2	0.04	97.57	93.40	48.83
P3508_3	47984637	47180036	14.4	14.2	0.04	97.54	93.33	48.98
P2334_4	48278892	47103113	14.5	14.1	0.04	97.42	93.11	49.22

Figure 23. Run quality control show excellent quality for all samples (Q20% and Q30% > 90)

Sample name	P2334_4	P2792_3	P2796_4	P2803J24	P3043_3	P3508_3	P3594_1	P3618_2	P3658_1	P3669_2	P3968_2	P3987_1
Total reads	94206226	94291286	77594184	100927458	115081358	94360072	91703410	90788376	104370672	75343246	87969154	90671504
Total mapped	89911901 (95.44%)	88434353 (93.79%)	74980671 (96.63%)	94465261 (93.60%)	108494289 (94.28%)	91260042 (96.71%)	88062031 (96.03%)	86782793 (95.59%)	100483761 (96.28%)	71831971 (95.34%)	84906108 (96.52%)	87135668 (96.10%)
Multiple mapped	3867150 (4.10%)	2937555 (3.12%)	2322340 (2.99%)	4130247 (4.09%)	7060998 (6.14%)	3093405 (3.28%)	2221410 (2.42%)	2098457 (2.31%)	2401547 (2.30%)	1766043 (2.34%)	2725900 (3.10%)	2493392 (2.75%)
Uniquely mapped	86044751 (91.34%)	85496798 (90.67%)	72658331 (93.64%)	90335014 (89.50%)	101433291 (88.14%)	88166637 (93.44%)	85840621 (93.61%)	84684336 (93.28%)	98082214 (93.97%)	70065928 (93.00%)	82180208 (93.42%)	84642276 (93.35%)
Read-1	43154313 (45.81%)	42897824 (45.50%)	36432334 (46.95%)	45548955 (45.13%)	50813147 (44.15%)	44257263 (46.90%)	43141383 (47.04%)	42510469 (46.82%)	49162563 (47.10%)	35066913 (46.54%)	41223103 (46.86%)	42380568 (46.74%)
Read-2	42890438 (45.53%)	42598974 (45.18%)	36225997 (46.69%)	44786059 (44.37%)	50620144 (43.99%)	43909374 (46.53%)	42699238 (46.56%)	42173867 (46.45%)	48919651 (46.87%)	34999015 (46.45%)	40957105 (46.56%)	42261708 (46.61%)
Reads map to '+'	42965483 (45.61%)	42663530 (45.25%)	36307598 (46.79%)	45135345 (44.72%)	50614260 (43.98%)	44054865 (46.69%)	42886036 (46.77%)	42322652 (46.62%)	48992327 (46.94%)	35014376 (46.47%)	41063204 (46.68%)	42274530 (46.62%)
Reads map to '-'	43079268 (45.73%)	42833268 (45.43%)	36350733 (46.85%)	45199669 (44.78%)	50819031 (44.16%)	44111772 (46.75%)	42954585 (46.84%)	42361684 (46.66%)	49089887 (47.03%)	35051552 (46.52%)	41117004 (46.74%)	42367746 (46.73%)
Non-splice reads	66994484 (71.11%)	63239698 (67.07%)	46421617 (59.83%)	72541546 (71.87%)	79771484 (69.32%)	59873893 (63.45%)	55284765 (60.29%)	56001672 (61.68%)	65635952 (62.89%)	47209604 (62.66%)	55626872 (63.23%)	57697832 (63.63%)
Splice reads	19050267 (20.22%)	22257100 (23.60%)	26236714 (33.81%)	17793468 (17.63%)	21661807 (18.82%)	28292744 (29.98%)	30555856 (33.32%)	28682664 (31.59%)	32446262 (31.09%)	22856324 (30.34%)	26553336 (30.18%)	26944444 (29.72%)

Figure 24. Mapping showing a very good quality for all samples in both reading direction

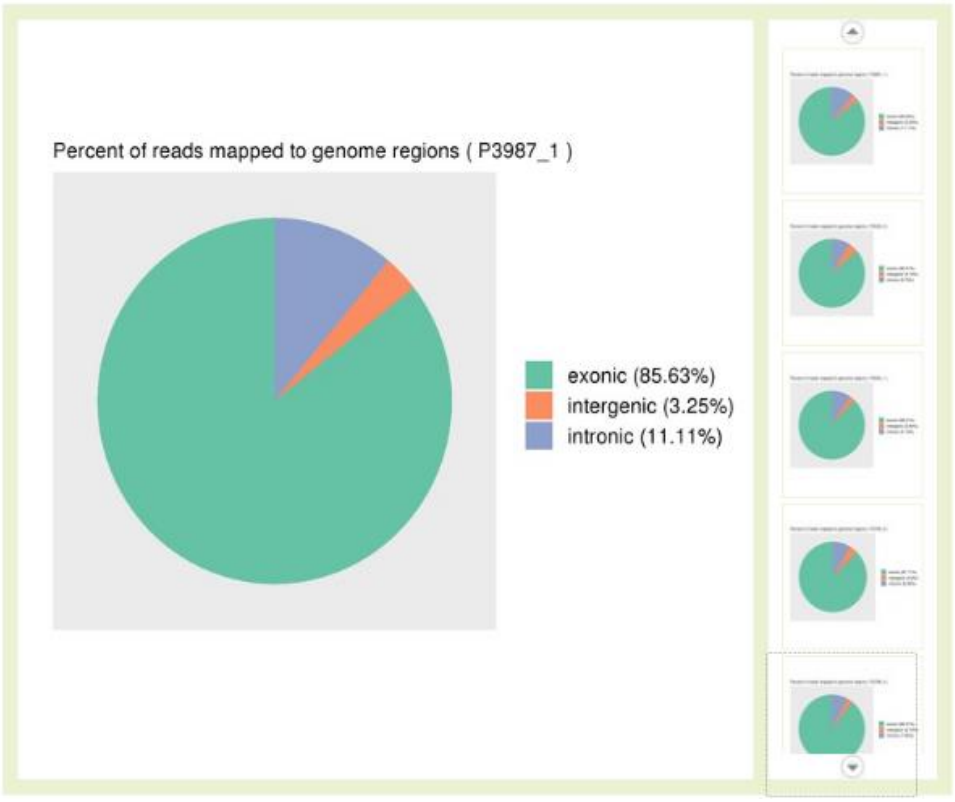

Figure 25. Exemple of qualitative mapping

Venn diagram (figure 26) analyzed co-expression of genes between groups and showed that 18353 genes were commonly expressed by all groups. The group of normal HCMV positive placental samples qualitatively expressed more genes than the normal HCMV negative group. For PE groups there were slight differences. The PE HCMV positive group showed small differenced when compared to normal HCMV positive group. These findings come to confirm our first observations about gene expression previously discussed in chapter 5.

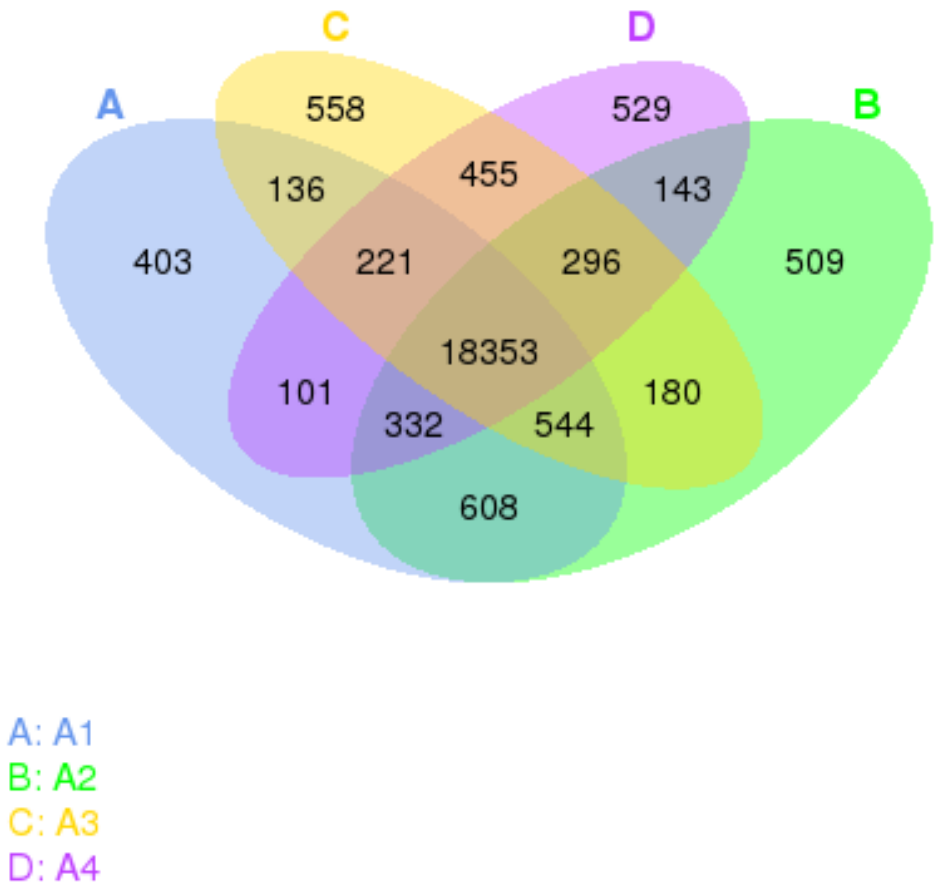

Figure 26. Venn diagram used to analyse the co-expression of genes.

Results' clusterisation shown an increased number of very well-expressed genes in all normal pregnancy samples compared to PE specimens (figure 27). The number of genes

analyzed in this step of our study is impressive and requires special training and time for a good interpretation. This interesting observation is still being processed at the time of writing this paper.

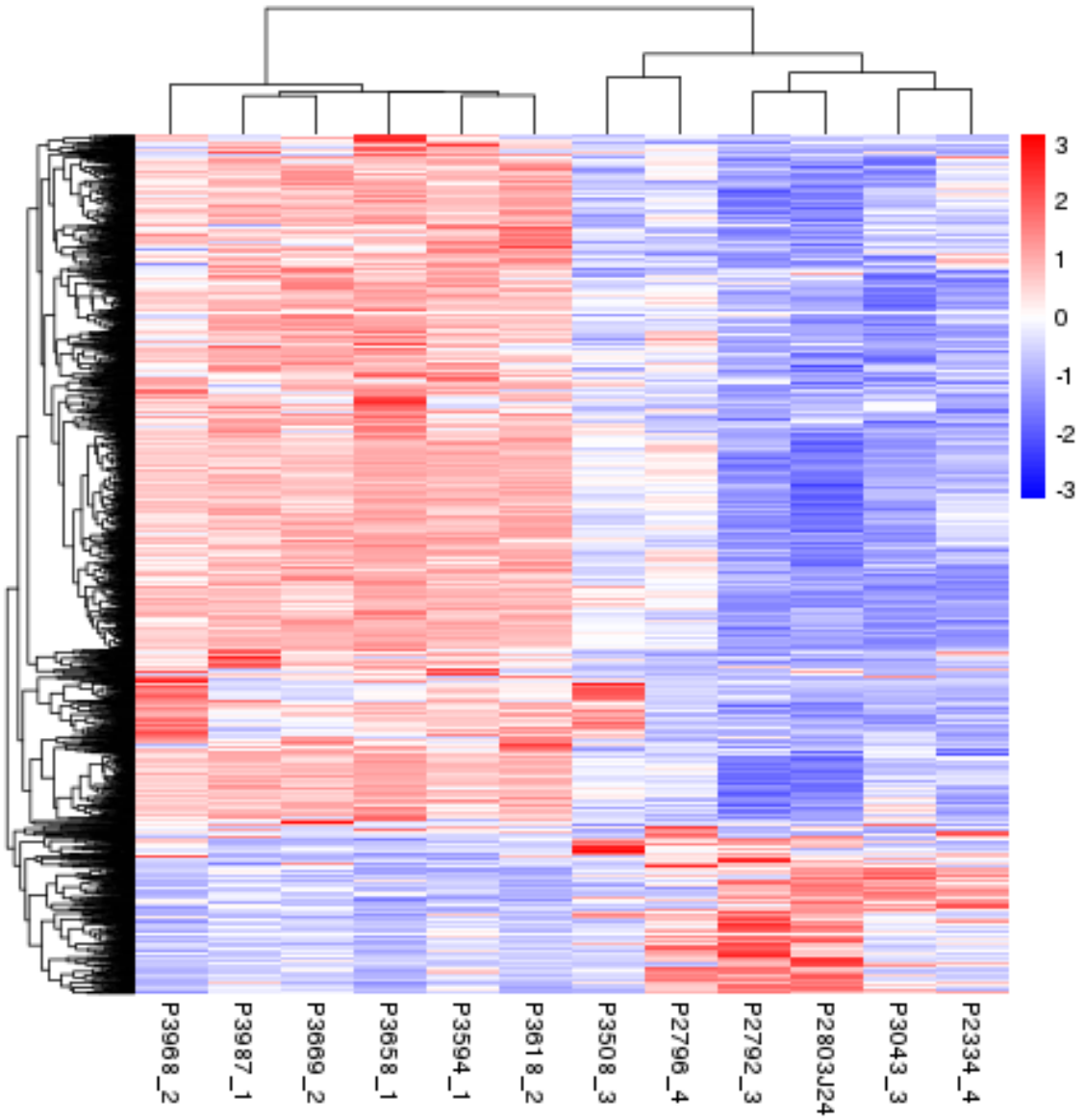

Figure 27. Gene expression clusterisation

The volcano plots made for these results are quite similar to our previous findings in Chapter 6. They suggest biologically and statistically significant differences for genes over-expression and under-expression in PE HCMV negative samples compared to normal HCMV negative samples, in all PE compared to normal HCMV negative samples and in normal HCMV positive group compared to normal HCMV negative group (figure 28).

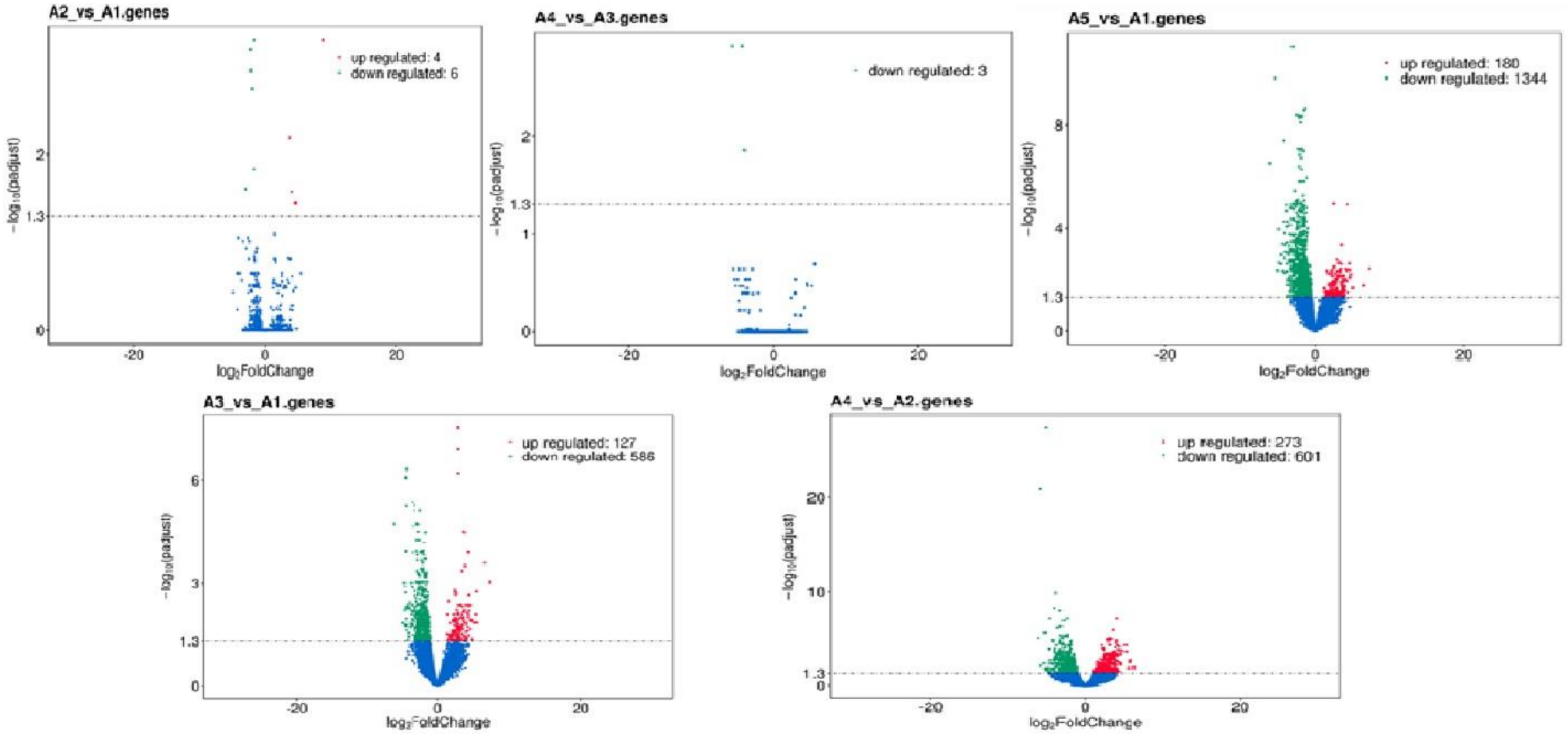

Figure 28. Volcano plots for transcriptomic quantitative analysis of gene expression in different tested groups

Another important observation was made by the enrichment analysis. This quantitative analysis aims to find out which biological functions or pathways are significantly associated with differential expressed genes. Thus, we could observe that no statistical differences were recorded between the two normal placental samples group nor between the PE groups (figure 29). However, genes involved in the immune system process, lipid binding and plasma membrane activity were significantly better expresses in all PE samples vs normal HCMV negative group as well as in PE HCMV negative group vs normal HCMV negative samples and PE HCMV positive group compared to normal HCMV positive specimens (figure 30).

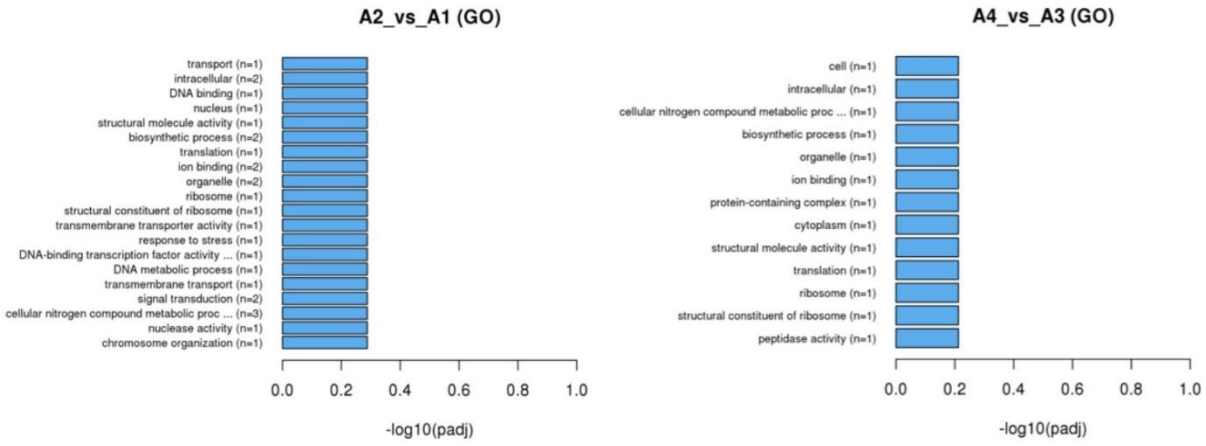

Figure 29. Enrichment analysis shows no statistical differences between normal pregnancy groups

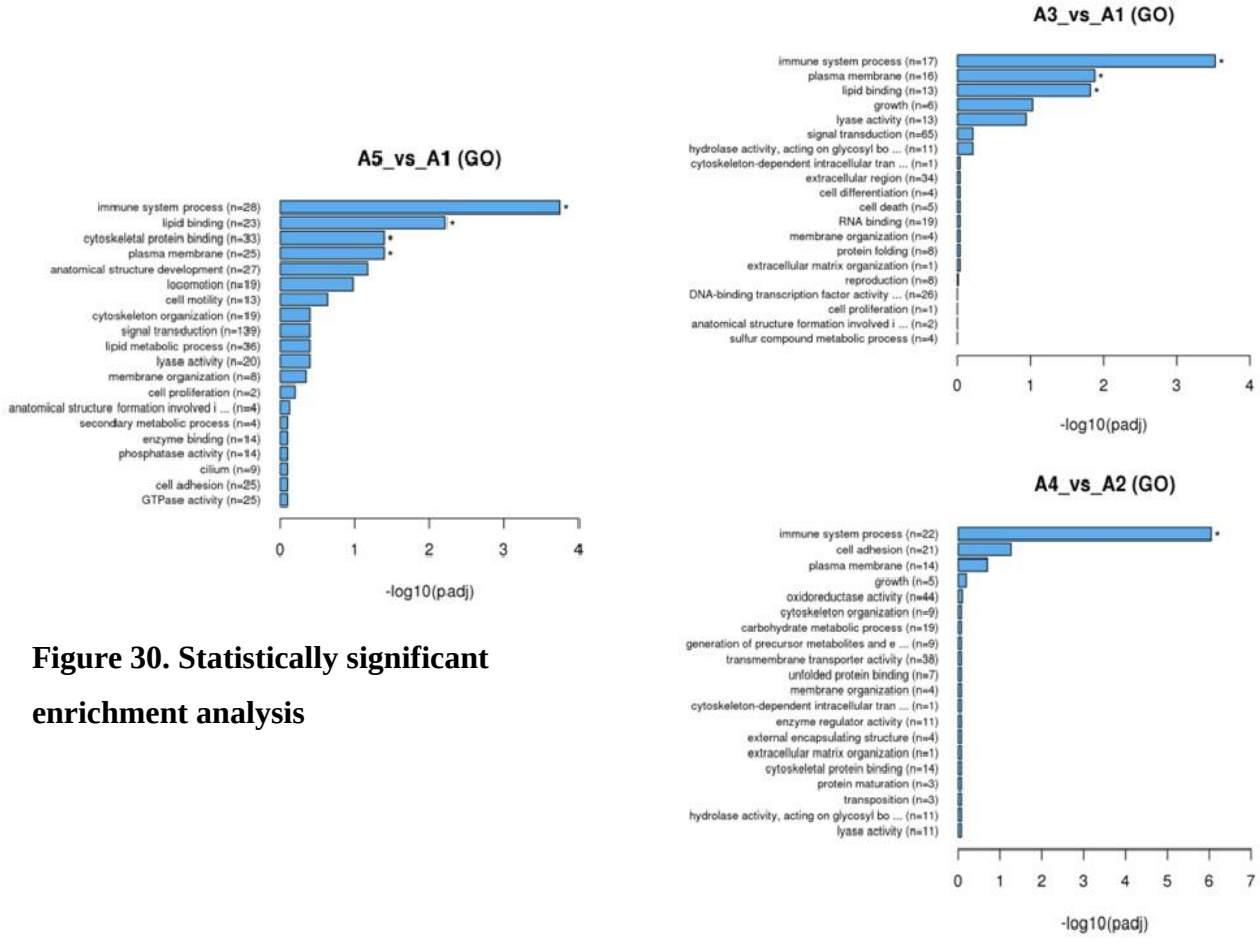

Figure 30. Statistically significant enrichment analysis

Even though no HCMV sequences were identified in this study, our transcriptome work is still in progress and will hopefully bring some answers about pro or anti-atherosclerotic genes expressed in preeclampsia. The lack of HCMV sequences does not totally exclude HCMV influence on vascular remodeling in preeclampsia in patients with latent infection. It is rather associated to non-active viral replication in our samples. All these genetic results are in preparation for submission as a full-length paper in *Viruses* MDPI Journal.

CHAPTER 7. FINAL DISCUSSIONS AND CONCLUSIONS

This project started with a clinical observation of an increased incidence of cardiovascular pathologies and cardiovascular related death in Romania. A little literature research in this direction showed a crucial impact of cardiovascular events on mortality and morbidity all over the world [31], despite all efforts of public health system to reduce the cardiovascular risk factors. Moreover, european statistics show that Romania has one of the highest rates of death related to cardiovascular disease in the European Union [120,143]. Infectious agents gained a special status in vascular remodeling during the last two decades, being more and more cited for their role in atherosclerosis, the underlying mechanism of cardiovascular disease. Cytomegalovirus has been the most intensively studied infectious agent due to the increasingly clear evidence of its role in the development of early lesions and the progression of atherosclerosis with cardiovascular events as an end point [40,85]. In this context we started our research by investigating the HCMV seroprevalence in Romania. To our knowledge, very few similar studies were developed until present and they were focused on a special population group, women of childbearing age [58,66]. Our results reveal a 92.9% of latent HCMV infection distribution within adult non-HIV infected group. HCMV is strongly related to poor outcomes in immunosuppressed patients. As most of the survivors of parenterally HIV infected children of the '80-90' HIV pandemic belong today to the adult population group we compared our HIV negative results to the HIV-positive group. No statistically significant difference between the two groups was identified, 92,7% in HIV positive patients vs 92,9% in HIV negative patient, p value 0.93. For an even greater relevance to our results, we added a 0 to 18 years old populational group. Luckily for this second assessment of HCMV seroprevalence in Romania we could join an international project from the French National Reference Center for Herpesviruses and compare our results to those from France and French territories, as HCMV seroprevalence seems to be influenced by the socio-economic status. There were significant differences for global seroprevalence, 55.3% in France compared to 92% in Romania. An increased prevalence after the age of two years is seen in Romania, although interestingly, in both countries the age of primo-infection is very low, under the age of 5 years old. We presume that an early CMV infection, with possible multiple reactivations during lifetime, triggers a chronic inflammatory process that is amplified in patients with associated risk factors (obesity, diabetes, smoking), increasing the severity of cardiovascular disease in Romania. To further evaluate this hypothesis, we investigated a possible correlation between anti HCMV antibody titers, as an expression of multiple viral

reactivations and the cardiovascular risk in a group of immunocompetent subjects, hospitalized for diverse reasons. Higher titers of anti HCMV IgG antibodies were associated to more important cardiac changes but did not correlate to an increased left ventricular mass. However, the small dimension of our cohort, as well as the unicentric character of our work, are some biases that need to be corrected in further studies for a better vison of the role of HCMV infection in cardiovascular disease in a country with a very high incidence of this infection and one of the highest mortality rates due to cardiovascular pathologies in Europe. In the HIV positive group, subjects with higher titers of IgG HCMV antibodies were those who had more severe immnuno-supression and higher viral loads during their disease (as shown by higher CD 4 nadir and VL zenith values), which made them prone for more frequent CMV reactivations. As for now, these subjects are still very young, and display no significant correlation between cardiovascular risk parameters (serum triglycerides, cholesterol, blood glucose, blood pressure values) and the level of anti CMV antibodies. Unfortunately, evaluation of the intima-media thickness was not available to assess an early increased risk of atherosclerosis, and this is an important future objective to be accomplished.

Besides its possible role in cardiovascular disease, HCMV is the main cause of neurosensory disorders in newborns and children when the infection is acquired or reactivated during pregnancy [28,32]. Within the placenta HCMV, was associated to vascular impairment [126] and possible development of hypertensive pregnancy disorders [38,164]. PE is a pregnancy condition that was incriminated as a possible risk factor for cardiovascular impairment later in life in both mother and child [4,12]. With this background data, we undertook a new research direction, aiming at to assess the dynamic action of HCMV on the vascular wall, in special risk groups, such as patients who developed preeclampsia during pregnancy. With the opportunity of an international research scholarship, we could assess the role of HCMV infection in women with normal pregnancies and preeclampsia. Thus, at the University of Limoges in the Inserm U1092 Unit, France, we designed a study that used one of the largest cohorts in literature, to our knowledge, of PE placental specimens to look for the role of HCMV in vascular remodeling and acute atherosis lesions. Our first observation was that cumulative action of latent HCMV infection, assessed by the presence of anti HCMV IgG antibodies, and BMI> 25 kg/2 or maternal age influences the severity of PE. Then, the HCMV positive serostatus of the mother highly influenced the low weight at birth. Courtesy to the Pathology and Obstetrics Departments of the Mother and Child Hospital CHU Limoges we analyzed a

collection of 72 frozen placental specimens, 52 from PE pregnancies and 20 from normal pregnancies. Histological findings suggest acute atherosis in some sections from obese HCMV positive patients with PE. However, interpreting these results was quite difficult due to a lack of experience with placental frozen specimens. Not only that there are not so many published studies about vascular remodeling in human placentas, but the majority used paraffin embedded blocks or sections that have a different regimen of staining techniques and interpretation. 12 samples were selected from the collection for genetic tests according to their quality and quantity of total RNA as well as for similar clinical characteristics. Thus, we could generate 4 different groups consisting each of 3 samples – 1 PE HCMV positive, 1 PE HCMV negative, 1 normal pregnancy HCMV positive and 1 normal pregnancy HCMV negative. Our first results on human atherosclerosis gene expression, using a commercial kit, revealed some biologically and statistically gene expression differences between the normal HCMV positive pregnancies and normal HCMV negative pregnancies and more important changes in PE HCMV positive specimens when compared to normal HCMV positive samples. However, the changes in gene expression between the two groups were quite heterogeneous and mainly targeted genes that encode for chemoattractant factors and adhesion molecules. In these conditions, for a better approach, we made a transcriptomic analysis for the same 12 samples as before, hoping for a particular HCMV signature. The results of transcriptomics showed an excellent total RNA quality of our samples after 4 different methods of evaluation, but no HCMV sequence was identified. This was unexpected as we included in our samples 2 PE with possible viral reactivation (equivocal and positive anti HCMV IgM antibodies). These findings do not have to be interpreted as a clear absence of HCMV. First, we know that HCMV distribution within the placenta is uneven and sometimes may rest only at the level of the spiral arteries without crossing the placental entire structure [126,149]. Thus, the biopsy samples that we used might have missed the infected spots. Second, there is no certain proof of viral reactivation in our samples, as no blood viral load was available and sometimes the presence of IgM antibodies may be due to cross-reactivity. This transcriptome analysis is extremely comprehensive and complicated and needs teamwork, experience and time for good interpreting. Therefore, at the moment of writing this paper the analysis of these results is still in progress. However, our first data suggest the most significant gene changes in PE versus normal pregnancies and between normal HCMV positive samples and normal HCMV negative samples as well as no significant differences between the 2 PE groups. These preliminary results are quite similar to those observed

with the gene expression assessed using a commercial kit, and they also point out to genes involved in cellular adhesion, plus some series of genes that encode for immune response. From what we know so far this is the first study to look at the villous vascular network and fetal interface of the human placenta in relation to vascular remodeling and gene expression.

Besides a very high seroprevalence of HCMV in Romania, probably the most important conclusion of this work is that all our results suggest an ancillary role of HCMV in vascular remodeling. For example, it may interfere with uterine vascular network leading to PE, but without having a direct influence on long term cardiovascular outcome of the newborn or the mother. The preeclamptic status itself may directly impact the long-term cardiovascular outcome by a mechanism that needs to be clarified. Hopefully, our final results from transcriptomic analysis will add some more informational bricks in this direction.

CHAPTER 8. FUTURE PERSPECTIVES

Our first future objective is to design a multicentric prospective study that will include a large cohort of patients of all group ages to better assess the seroprevalence of HCMV in Romania. All these patients should be examined and evaluated for cardiovascular risk and cardiovascular disease and HCMV viral load should be added in their blood tests for a better correlation of the infection to the severity of cardiovascular disease. Another aim is to ask the patients that we included in our first seroprevalence study for a reevaluation to check for any HCMV reactivation related to the cardiovascular disease progression and to longitudinally monitor the HIV infected patients for early signs of atherosclerosis and cardiovascular risk.

A second aim is to check for the HCMV presence within the atheroma plaques from carotid, femoral and pulmonary arteries that we collected in Bucharest, Romania, at the beginning of the project, but remained untested due to work reorientation during the years of COVID 19 pandemic, an already high volume of work, as well as a lack of financial resources.

As all the protocols were designed and verified during this part of the project, we would like to create a prospective cohort of placental samples, this time using more biopsies from the same placenta, having a viral load confirmation for each patient and collect frozen, as well as paraffin embedded samples. Uterine biopsies, although very difficult to obtain, would represent valuable additions to compare the changes between the maternal and fetal face of the placenta.

LIST OF PUBLISHED ARTICLES AND CONGRESS PARTICIPATION

In extenso articles:

1. **THE ROLE OF CMV INFECTION IN PRIMARY LESIONS, DEVELOPMENT AND CLINICAL EXPRESSION OF ATHEROSCLEROSIS, Cristescu CV**, Alain S, Ruță SM.. J Clin Med. 2022 Jul 1;11(13):3832. doi: 10.3390/jcm11133832. PMID: 35807114; PMCID: PMC9267753.

2. **CYTOMEGALOVIRUS INFECTION AND CARDIOVASCULAR RISK IN A MONOCENTRIC ROMANIAN ADULT PATIENT GROUP**, **Cristescu CV**, Ghica M, Udeanu DI, Amzar AI, Dita C, Grancea C, Siliste R, Vintila AM, Homentcovschi C, Alain S, Ruta SM, *FARMACIA* (2022), Vol 70,2, DOI:10.31925/farmacia.2022.2.16, Corpus ID: 250565752

Congress Posters/Abstracts

1. **Heart Failure Congress 2017, Paris, France**
   P1974 - The impact of chronic cytomegalovirus infection on left ventricular hypertrophy, R Siliste, **C Cristescu**, C Dita, AM Vintila, C Grancea, S Ruta, European Journal of Heart Failure (2017) **19** (Suppl. S1) 5–601, doi:10.1002/ejhf.833

2. **Congress of the University of Pharmacy and Medicine "Carol Davila", Bucharest, Romania, 2017**
   (ID 323) Correlation of high seric levels of anti-cytomegalovirus IgG antibodies with cardiovascular disease, C. Cristescu, M. Anton, C. Homentcovschi, R. Siliste, A. Vintila,C. Dita, C. Grancea, S. Ruta, Maedica A Journal of Clinical Medicine, Volume 12 (15) Supplement 2017, ISSN 1841-9038, e-ISSN 2069-6116, ISSN-L 1841-9038

3. **Translational Research Updates In Virology, Cajal Congress of the Romanian Academy 2017, Bucharest, Romania - Comparative study on cytomegalovirus seroprevalence in positive HIV adult population versus negative HIV adult population in Romania, Carmen Cristescu**, Camelia Grancea, Adelina Rosca, Luminita Ene, Simona Ruta

SUMMARY

INTRODUCTION

Acute ischemic heart disease and stroke are the two main causes of death from cardiovascular causes worldwide. In the European Union, despite all the public health programs, mortality and morbidity associated with cardiovascular causes remained high in the last 10 years. Romania is among the leading countries in Europe in terms of the number of deaths related to a heart attack or stroke. Cardiovascular disease has a long-term evolution that may be precipitated or aggravated by the long term action of infectious agents such as human cytomegalovirus, in addition to conventional risk factors such as obesity, smoking, hypertension and diabetes, implies the action of other pathologies such as hypertensive pregnancy disorders.

AIMS

The main goal of this study was to evaluate a possible connection between human cytomegalovirus infection and the early development and progression of cardiovascular diseases. The specific objectives were:

3. To evaluate the cytomegalovirus seroprevalence in the Romanian population, in different age and risk groups, and to investigate a possible correlation between anti-HCMV antibody titers, as an expression of multiple viral reactivations, and the cardiovascular risk.
4. To assess the dynamic action of HCMV on the vascular wall, using the expression of proinflammatory cytokines and growth factors, in special risk groups, such as patients who developed preeclampsia during pregnancy, due to the emerging evidence of an increased risk of progression towards cardiovascular disease in this particular risk group. To gain a mechanistic view of this pathology, in collaboration with the National Reference Center for Cytomegalovirus in Limoges, France, we set out to investigate particular aspects related to HCMV-mediated vascular remodeling and the expression of various genes with a role in atherosclerosis within the placenta.

MATERIALS AND METHODS

To assess the human cytomegalovirus seroprevalence in Romania, a total of 422 patients aged 19 to 79, mean age of 47.79 years old were enrolled in this study. Of these, 228 patients, mean age of 22.15 years old, were included retrospectively from an HIV-

positive cohort, available at the Stefan S Nicolau National Institute of Virology in Bucharest, Romania. This cohort included HIV-treated patients admitted between 2012-2016 from several departments of infectious diseases in Bucharest. The rest of the 194 patients, mean age of 55.5 years old, were prospectively enrolled from the general population, from the internal medicine department of the Coltea Clinical Hospital in Bucharest, Romania, between 2016-2017. To carry out this study, all legal and ethical provisions in force were respected. All patients included in this study signed a written consent form, agreeing to the collection of blood samples as well as data from their medical records and their processing for research purposes. The data obtained from all 422 patients were used exclusively for questioning HMCV seroprevalence and studying the distribution of this infection in the HIV-positive population and the general population. This part of our research was partially funded by an internal grant project, PFE_23/2018, from the University of Medicine and Pharmacy "Carol Davila", Bucharest. For an even greater relevance to our results, we had the opportunity of international collaboration and we added a 0 to 18 years old populational group to have a global perspective on the viral distribution within the Romanian population. In addition to seroprevalence, this cohort was also used to assess the association of the anti-CMV IgG antibody titer with left ventricular hypertrophy and the severity of the cardiovascular disease. Left ventricular mass (grams) and left ventricular mass indexed to total body surface area were determined using the Devereaux modified formula, 0.8{1.04[([LVEDD + IVSd +PWd]3 - LVEDD3)]} + 0.6. The severity of cardiac disease was assessed using AHA and ESC guidelines as well as New York Heart Association (NYHA) functional classification:

0 no cardiac changes

1 light cardiac changes - NYHA I

2 mild cardiac changes – NYHA II

3 moderate to severe cardiac changes - NYHA III - NYHA IV

4 recent history or acute indication of minimally invasive or surgical procedures for cardiovascular disease

The presence of anti-HCMV IgM and IgG antibodies was assed for all patients included in this study at Stefan S Nicolau National Institute of Virology, Bucharest, Romania, using a quantitative enzyme immunoassay (DiaPro, Diagnostic Bioprobes SRL, Italy). According to the manufacturer's instructions, a sample with a reactivity over 0.5 UI/L was considered positive. The patients from the prospective cohort were divided into two groups based on the median value of reactivity for the CMV positive samples = low CMV IgG titers (0.5-5

UI/L) and high CMV IgG titers (>5UI/L). ELISA screening tests for HIV were also performed in all patients. For HIV positive cohort the quantitative determination of viral nucleic acid by RT-PCR (present and zenith value) and the CD4 cell number (present and nadir) was also available. The obtained data were processed statistically with the help of Windows 10 office Excel and R software 4.1.1.

To evaluate the possible action of human cytomegalovirus in vascular remodeling and severity of preeclampsia, fifty-two women of fertile age, mean age of 33.15 years old (18 to 47 years old) who developed preeclampsia during pregnancy were enrolled retrospectively in this study. A control group of twenty patients with normal pregnancies, mean age of 29.89 years old (22 to 37 years old) was prospectively created. All legal and ethical provisions in force were respected for this study. All patients signed an informed consent by which they agreed to the processing of their biological samples and medical record for research purposes. All patients included in this study came from the Gynecology and Obstetrics Department of CHU Dupuytren Hospital, Limoges, France. With the support of this department and that of the Pathology Department from the same hospital, we collected information from patients' medical records as well as placental specimens and blood samples.

We searched the patients' ***medical record*** for the mother's age, the number of pregnancies, the method of obtaining the pregnancy, any medical history before pregnancy (autoimmune diseases, HIV, hepatitis, kidney disease, any cardiovascular or oncologic pathology), cardiovascular risk factors (hypertension, diabetes, smoking, obesity, dyslipidemia), gestational age at birth, newborn's weight, infections during pregnancy, and for the test group, the moment of the onset of preeclampsia. All data were processed using R open software 4.1.1.

Blood samples were collected from each woman during the first pregnancy medical examination and at delivery. For the test group, we used a sera collection from the CHU Dupuytren Mother and Child Hospital (CRBioLim biobank certified NFS96-900). For the control group, fresh blood samples were used. To determine specific anti-HCMV IgM and IgG antibodies a LIAISON XL automated chemiluminescence analyzer was used. All patients were also screened for HIV and hepatitis virus B and C. No HIV, nor the hepatitis-infected patient was included in the study.

Placental specimens were obtained for the test group from the CHU Dupuytren Pathology collection (2016-2018) – frozen specimens kept at -80*C. For the control group, I strictly

followed the collection protocol of the Pathology Department. Thus, the entire fresh placenta was collected after birth and kept at 4*C before processing (no later than 72 hours). A full thickness "carrot" biopsy was taken and immersed in liquid nitrogen for 5 seconds, then immediately transferred to dry ice and then stored in the shortest possible time at -80*C.

Expression of genes involved in atherosclerosis

Placental specimens were used to analyse and compare the expression of specific atherosclerosis genes. Thus, 4 different groups were designed, each of them containing three samples:

- Normal Pregnancy HCMV negative
- Normal Pregnancy HCMV positive
- PE HCMV negative
- PE HCMV positive

Each frozen sample was sectioned with a cryotome at five different levels. 20 slides of 10µm each were then stored in 1 ml of trizole, TRI Reagent® RNA Isolation Reagent for carrying out ARN extraction. After 5 minutes of dissociation 0.2 ml of cold (-20*C) chloroform was added. The tubes were vigorously stirred by hand for 15 seconds and then left at room temperature for 2-3 minutes. Afterwards, a centrifugation was performed at 11200 rpm, at 4*C, for 15 minutes. Then, the supernatant was transfered to a new tube. 600 µl of room temperature 70% ethanol was added and mixed by pipetting. Further, the mixture was transferred to a working column and the extraction continued according to the standard protocol of the QIAmp® RNA Blood Mini Kit. At the end of the extraction each sample was tested for RNA quality using NanoDrop spectophotometer measurements and RNA electrophoresis using a standard Agilent® RNA 6000 Nano kit. The samples used for gene expression identification had a similar good RNA quality. Furthermore, cDNA was synthetized using Qiagen RT^2 First Strand® Kit. The cDNA was then amplified with the real-time RT^2 Profiler PCR Array (QIAGEN, Cat. no. PAHS-038Z) in combination with RT^2 SYBR® Green qPCR Mastermix (Cat. no. 330529). Ct values were exported to an Excel file to create a table of Ct values. This table was then uploaded on to the data analysis web portal at http://www.qiagen.com/geneglobe. This data analysis report was exported from the QIAGEN web portal at GeneGlobe.

To asses gene expression we compared the following groups os samples:

4. **Normal HCMV POSITIVE** placenta (test group) to **Normal HCMV NEGATIVE** placenta (control group)
5. **POSITIVE HCMV PE** placenta (test group) to **NEGATIVE HCMV PE** placenta (control group)
6. **POSITIVE HCMV PE** placenta (test group) to **Normal POSITIVE HCMV** placenta (control group)

Histopathological analysis

Frozen sections of 5 µm from placental biopsies were done using a cryotome. They were fixed in cold pure acetone solution (90%) for 20 minutes. Haematoxylin-eosin-saffron (HES) staining was automatically done in order to look for the detachment of the arterial intima. The stained sections were analyzed with a digital slide scanner, Hamamatsu® NanoZoomer and the NDP viewer software.

Immunostaining was also performed in order to look for HCMV presence within placental blood vessels. Multiple markers were targeted with an indirect immunofluorescence protocole. The targets were endothelial cells (CD31), smooth muscle cells (alpha actin smooth muscle), macrophages (CD 68), HCMV IE antigens and DAPI. The antibodies used for immunofluorescence technique and the dilutions are shown in table 1.

Table 1. Antibodies and dilutions used for immunostaining

Target	Unconjugated antibodies	Dilution	Conjugated antibodies	Dilution
EC	-	-	**Ozyme SigmaAldrich** BLE303126, Alexa Fluor 594 anti-human CD31	1/50
Macrophages	-	-	**Abcam** Recombinant Alexa Fluor® 647 Anti-CD68 antibody [EPR20545] (ab224029)	1/50
SMC	-	-	**Termo-Fischer** 25UG AntiAlpha-	1/1500

			Smooth Muscle Actin eFluor 570	
HCMV IE antigen	**Abcam** Anti-CMV IE1 and IE2 antibody [CH160] (ab53495)	1/50	**Abcam** Goat Anti-Mouse IgG Alexa Fluor® 488 (ab150113)	1/200
Nucleus	Abcam DAPI Staining Solution (ab228549)	1/1000	-	

Fixed frozen sections were washed out with PBS solution 3 times for 5 minutes. Further more cell permeabilization was done with PBS-Triton 0.1% for 5 minutes at room temperature. Another washout was performed with PBS 3 times for 5 minutes. Then, non-specific sites were fixed with PBS-BSA 0.5% for 30 minutes at room temperature. Further, 50 µl of unconjugated mouse anti-HCMV IE antigens antibodies (dilution 1/50) were added to sections followed by overnight incubation at 4 degrees. The next day a washout was done using a stirring platform – 3 times for 10 minutes. Next, 1500 µl mix of conjugated anti-CD31, anti-CD68, anti-SMC and anti-HCMV IEA (goat anti-mouse) was obtained, respecting the above-mentioned dilutions for each specific antibody. This mix was then added to sections and incubated for one hour at 37 degrees. After this, a new washout -3 times x 10 minutes was done. The next step was DAPI staining with 1/1000 DAPI solution dilution for 5 minutes in a dark place, at room temperature. The last washout – 3 times x5 minutes was performed using PBS solution. In the end, the sections were fixed with a special liquid and varnished. Before confocal microscope analysis, all sections were stored away from light at 4 degrees. For analysis, a confocal Carl Zeiss Zen2 microscope was used. The image acquisition and processing were performed with ImageJ software, at the confocal microscopy platform (Biscem Inserm platform, Limoges University).

RESULTS

Seroprevalence of latent HCMV infection, highlighted by the presence of specific IgG anti-HCMV antibodies was comparable in the general population and positive HIV

patients. There were no statistical differences, between the two groups, 92,7% in HIV-positive patients vs 92,9% in HIV-negative patients, p-value of 0.93. We compared our results to those from France and French territories. There were significant differences in global seroprevalence, 55.3% in France compared to 92%. Interestingly, in both countries the age of infection is very low, under the age of 5 years old. In the HIV positive group, subjects with higher titers of IgG HCMV antibodies (> 8.76 IU / ml) had significantly lower values of the current number of CD4 cells (367 vs. 523, p = 0.02), of the CD4 /CD8 ratio (0.39 *vs* 0.74, p = 0.01) and of the number of CD4 nadir (45 vs 143, p = 0.003), as well as significantly higher values of zenith HIV RNA (5.3 vs. 4.2 copies/ml, p = 0.001) (figure 10). There was no significant correlation between cardiovascular risk parameters (serum triglycerides, cholesterol, blood glucose, blood pressure values) and the level of anti CMV antibodies. Unfortunately, assessment of the left ventricular function or evaluation of the intima-media thickness was not available to assess an early increased risk of atherosclerosis. A prospective study looking for these aspects will bring additional important information on the clinical risk for cardiovascular diseases in this vulnerable group of patients. We presume that an early CMV infection, with possible multiple reactivations during lifetime, triggers a chronic inflammatory processs that is amplified in patients with associated risk factors (obesity, diabetes, smoking), increasing the severity of cardiovascular disease in Romania. To further evaluate this hypothesis, we investigated a possible correlation between anti-HCMV antibody titers, as an expression of multiple viral reactivations and the cardiovascular risk in a group of immunocompetent subjects, hospitalized for diverse reasons. Higher titers of anti-HCMV IgG antibodies were associated to more important cardiac changes but did not correlate to an increased left ventricular mass. However, the small dimension of our cohort, as well as the unicentric character of our work, are some biases that need to be corrected in further studies for a better vision of the role of HCMV infection in cardiovascular disease in a country with a very high incidence of this infection and one of the highest mortality rates due to cardiovascular pathologies in Europe. In the HIV-positive group, subjects with higher titers of IgG HCMV antibodies were those who had more severe immune-supression and higher viral loads during their disease (as shown by higher CD 4 nadir and VL zenith values), which made them prone to more frequent CMV reactivations. As for now, these subjects are still very young and display no significant correlation between cardiovascular risk parameters (serum triglycerides, cholesterol, blood glucose, and blood pressure values) and the level of anti-CMV antibodies. Unfortunately, evaluation of the intima-media thickness

was not available to assess an early increased risk of atherosclerosis, and this is an important future objective to be accomplished.

Human cytomegalovirus influences in preeclampsia

Besides its possible role in cardiovascular disease, HCMV is the main cause of neurosensory disorders in newborns and children when the infection is acquired or reactivated during pregnancy [28,32]. Within the placenta HCMV, was associated to vascular impairment [126] and possible development of hypertensive pregnancy disorders [38,164]. PE is a pregnancy condition that was incriminated as a possible risk factor for cardiovascular impairment later in life in both mother and child [4,12]. With this background data, we undertook a new research direction, aiming at to assess the dynamic action of HCMV on the vascular wall, in special risk groups, such as patients who developed preeclampsia during pregnancy. With the opportunity of an international research scholarship, we could assess the role of HCMV infection in women with normal pregnancies and preeclampsia. Thus, at the University of Limoges, France, we designed a study that used one of the largest cohorts in literature, to our knowledge, of PE placental specimens to look for the role of HCMV in vascular remodeling and acute atherosis lesions. Our first observation was that cumulative action of latent HCMV infection, assessed by the presence of anti HCMV IgG antibodies, and BMI> 25 kg/2 or maternal age influences the severity of PE. Then, the HCMV positive serostatus of the mother highly influenced the low weight at birth. Courtesy to the Pathology Department of the Mother and Child CHU Limoges we analyzed a collection of 72 frozen placental specimens, 52 from PE pregnancies and 20 from normal pregnancies. For the test group, out of 52 patients included in this study, 33 (63%) had latent HCMV infection. There were no statistically significant differences between the HCMV-positive PE women and the negative ones in terms of early onset of severe preeclampsia incidence, nor in the presence of risk factors like in vitro fertilization, hypertension, presence of gestational diabetes, BMI >25kg/m2, smoking. Only multiple pregnancies were statistically significant in the HCMV positive group compared to the negative one (p-value 0.001). Histological findings suggest acute atherosis in some sections from obese HCMV positive patients with PE. However, interpreting these results was quite difficult due to a lack of experience with placental frozen specimens. Not only that there are not so many published studies about vascular remodeling in human placentas, but the majority used paraffin embedded blocks or sections that have a different regimen of staining techniques and interpretation. 12

samples were selected from the collection for genetic tests according to their quality and quantity of total RNA as well as for similar clinical characteristics. Thus, we could generate 4 different groups consisting each of 3 samples – 1 PE HCMV positive, 1 PE HCMV negative, 1 normal pregnancy HCMV positive and 1 normal pregnancy HCMV negative. Our first results on human atherosclerosis gene expression, using a commercial kit, revealed some biologically and statistically gene expression differences between the normal HCMV positive pregnancies and normal HCMV negative pregnancies and more important changes in PE HCMV positive specimens when compared to normal HCMV positive samples. However, the changes in gene expression between the two groups were quite heterogeneous and mainly targeted genes that encode for chemoattractant factors and adhesion molecules.

For a better approach, we made a transcriptomic analysis for the same 12 samples as before, hoping for a particular HCMV signature. The results of transcriptomics showed an excellent total RNA quality of our samples after 4 different methods of evaluation, but no HCMV sequence was identified. This was unexpected as we included in our samples 2 PE with possible viral reactivation (equivocal and positive anti HCMV IgM antibodies). These findings do not have to be interpreted as a clear absence of HCMV. First, we know that HCMV distribution within the placenta is uneven and sometimes may rest only at the level of the spiral arteries without crossing the placental entire structure [126,149]. Thus, the biopsy samples that we used might have missed the infected spots. Second, there is no certain proof of viral reactivation in our samples, as no blood viral load was available and sometimes the presence of IgM antibodies may be due to cross-reactivity. This transcriptome analysis is extremely comprehensive and complicated and needs teamwork, experience and time for good interpreting. Therefore, at the moment of writing this paper the analysis of these results is still in progress. However, our first data suggest the most significant gene changes in PE versus normal pregnancies and between normal HCMV positive samples and normal HCMV negative samples as well as no significant differences between the 2 PE groups. These preliminary results are quite similar to those observed with the gene expression assessed using a commercial kit, and they also point out to genes involved in cellular adhesion, plus some series of genes that encode for immune response. From what we know so far this is the first study to look at the villous vascular network and fetal interface of the human placenta in relation to vascular remodeling and gene expression.

CONCLUSIONS

Our data suggest a very high seroprevalence of human cytomegalovirus in the Romanian population, more than 90%, with very early acquisition of the infection, before the age of 5 years old. In our study, the severity of cardiovascular disease correlated to higher titers of anti-human cytomegalovirus IgG antibodies but did not influence the left ventricular mass. Evaluation of the atherosclerotic gene profile show important changes in gene expression between preeclampsia human cytomegalovirus-positive samples and normal human cytomegalovirus-positive specimens, as well as between normal human cytomegalovirus-positive samples compared to normal human cytomegalovirus-negative once.

By corroborating all the data obtained as a result of this work we can state that human cytomegalovirus is rather a co-factor in the initiation and development of cardiovascular diseases. However, taken together, all our results suggest an ancillary role of HCMV in vascular remodeling. For example, it may interfere with uterine vascular network leading to PE, but without having a direct influence on long term cardiovascular outcome of the newborn or the mother. The preeclamptic status itself may directly impact the long-term cardiovascular outcome by a mechanism that needs to be clarified. Hopefully, our final results from transcriptomic analysis will add some more informational bricks in this direction.

Further extensive studies on larger cohorts of patients should be performed in order to answer more complex questions about the role and the mechanism of action of human cytomegalovirus in cardiovascular pathology.

Résumé

INTRODUCTION

Les cardiopathies ischémiques aiguës et les accidents vasculaires cérébraux sont les deux principales causes de décès d'origine cardiovasculaire dans le monde. Dans l'Union européenne, malgré tous les programmes de santé publique, la mortalité et la morbidité associées aux causes cardiovasculaires sont restées élevées au cours des 10 dernières années. La Roumanie est parmi les premiers pays d'Europe en termes de nombre de décès liés à une crise cardiaque ou à un accident vasculaire cérébral. Les maladies cardiovasculaires ont une évolution à long terme qui peut être précipitée ou aggravée par l'action à long terme d'agents infectieux tels que le cytomégalovirus humain, en plus des facteurs de risque classiques tels que l'obésité, le tabagisme, l'hypertension et le diabète, ou l'action d'autres pathologies telles que les troubles hypertensifs de la grossesse.

OBJECTIFS

L'objectif principal de cette étude était d'évaluer un lien possible entre l'infection à cytomégalovirus humain et le développement précoce et la progression des maladies cardiovasculaires. Les objectifs spécifiques étaient :

1. Évaluer la séroprévalence du cytomégalovirus dans la population roumaine, dans différents groupes d'âge et de risque, et rechercher une éventuelle corrélation entre les titres d'anticorps anti-HCMV, en tant qu'expression de multiples réactivations virales, et le risque cardiovasculaire.
2. Évaluer l'action dynamique du HCMV sur la paroi vasculaire, en utilisant l'expression de cytokines pro-inflammatoires et de facteurs de croissance, dans des groupes à risque particuliers, tels que les patientes ayant développé une prééclampsie pendant la grossesse, en raison des preuves émergentes d'un risque accru de progression vers maladies cardiovasculaires dans ce groupe à risque particulier. Pour avoir une vision sur le mécanisme de cette pathologie, en collaboration avec le Centre National de Référence des Cytomégalovirus de Limoges, France, nous avons entrepris d'étudier des aspects particuliers liés au remodelage vasculaire médié par le HCMV et à l'expression de divers gènes jouant un rôle dans l'athérosclérose au sein du placenta.

MATÉRIAUX ET MÉTHODES

Pour évaluer la séroprévalence du cytomégalovirus humain en Roumanie, un total de 422 patients âgés de 19 à 79 ans, âge moyenne de 47,79 ans, ont été inclus dans cette étude. Parmi ceux-ci, 228 patients, âge moyenne de 22,15 ans, ont été inclus rétrospectivement à

partir d'une cohorte séropositive, disponible à l'Institut National de Virologie Stefan S Nicolau à Bucarest, Roumanie. Cette cohorte comprenait des patients traités pour le VIH admis entre 2012 et 2016 dans plusieurs services de maladies infectieuses à Bucarest. Le reste des 194 patients, âge moyenne de 55,5 ans, ont été recrutés de manière prospective à partir de la population générale, du service de médecine interne de l'Hôpital Clinique Coltea de Bucarest, en Roumanie, entre 2016 et 2017. Pour mener à bien cette étude, toutes les dispositions légales et déontologiques en vigueur ont été respectées. Tous les patients inclus dans cette étude ont signé un formulaire de consentement écrit, acceptant le prélèvement d'échantillons sanguins ainsi que les données de leurs dossiers médicaux et leur traitement pour la recherche. Les données obtenues sur l'ensemble des 422 patients ont été utilisées exclusivement pour interroger la séroprévalence du HMCV et étudier la distribution de cette infection dans la population séropositive et la population générale. Cette partie de notre recherche a été partiellement financée par un projet de subvention interne, PFE_23/2018, de l'Université de Médecine et de Pharmacie "Carol Davila", Bucarest. Pour une pertinence encore plus grande de nos résultats, nous avons eu l'opportunité d'une collaboration internationale et nous avons ajouté un groupe populationnel de 0 à 18 ans pour avoir une perspective globale sur la distribution virale au sein de la population roumaine. Outre la séroprévalence, la cohorte prospective a également été utilisée pour évaluer l'association du titre d'anticorps IgG anti-CMV avec l'hypertrophie ventriculaire gauche et la sévérité de la maladie cardiovasculaire. La masse ventriculaire gauche (grammes) et la masse ventriculaire gauche indexée sur la surface corporelle totale ont été déterminées à l'aide de la formule modifiée de Devereaux, 0,8{1,04[([LVEDD + IVSd +PWd]3 - LVEDD3)]} + 0,6. La gravité de la maladie cardiaque a été évaluée à l'aide des directives de l'AHA et de l'ESC ainsi que de la classification fonctionnelle de la New York Heart Association (NYHA) :

0 aucun changement cardiaque

1 changements cardiaques légers - NYHA I

2 changements cardiaques légers/ modérés – NYHA II

3 changements cardiaques modérés à sévères - NYHA III - NYHA IV

4 antécédents récents ou indication aiguë de procédures mini-invasives ou chirurgicales pour maladie cardiovasculaire

La présence d'anticorps anti-HCMV IgM et IgG a été évaluée pour tous les patients inclus dans cette étude à l'Institut national de virologie Stefan S Nicolau, Bucarest, Roumanie, à l'aide d'un dosage immunoenzymatique quantitatif (DiaPro, Diagnostic Bioprobes SRL,

Italie). Selon les instructions du fabricant, un échantillon avec une réactivité supérieure à 0,5 UI/L était considéré comme positif. Les patients de la cohorte prospective ont été divisés en deux groupes sur la base de la valeur médiane de la réactivité pour les échantillons CMV positifs = faibles titres d'IgG CMV (0,5-5 UI/L) et titres élevés d'IgG CMV (> 5UI/L). Des tests ELISA de dépistage du VIH ont également été réalisés chez tous les patients. Pour la cohorte séropositive pour le VIH, la détermination quantitative de l'acide nucléique viral par RT-PCR (valeur actuelle et zénithale) et le nombre de cellules CD4 (présent et nadir) étaient également disponibles. Les données obtenues ont été traitées statistiquement à l'aide du logiciel Windows 10 Office Excel et R 4.1.1 software.

Pour évaluer l'action possible du cytomégalovirus humain sur le remodelage vasculaire et la sévérité de la prééclampsie, cinquante-deux femmes en âge de procréer, d'âge moyen de 33,15 ans (18 à 47 ans) ayant développé une prééclampsie au cours de la grossesse ont été incluses rétrospectivement dans cette étude. Un groupe témoin de vingt patientes ayant des grossesses normales, d'âge moyen de 29,89 ans (22 à 37 ans) a été prospectivement constitué. Toutes les dispositions légales et déontologiques en vigueur ont été respectées pour cette étude. Tous les patients ont signé un consentement éclairé par lequel ils acceptaient le traitement de leurs échantillons biologiques et de leur dossier médical à des fins de recherche. Tous les patients inclus dans cette étude provenaient du service de Gynécologie et d'obstétrique de l'hôpital CHU Dupuytren, Limoges, France. Avec le soutien de ce service et celui du service de Pathologie du même hôpital, nous avons collecté des informations à partir des dossiers médicaux des patients ainsi que des prélèvements placentaires et des prélèvements sanguins.

Nous avons recherché dans le dossier médical des patientes l'âge de la mère, le nombre de grossesses, le mode d'obtention de la grossesse, les éventuels antécédents médicaux avant la grossesse (maladies auto-immunes, VIH, hépatite, maladie rénale, toute pathologie cardiovasculaire ou oncologique), les facteurs de risque cardiovasculaire (hypertension, diabète, tabagisme, obésité, dyslipidémie), âge gestationnel à la naissance, poids du nouveau-né, infections pendant la grossesse, et pour le groupe test, moment du début de la prééclampsie. Toutes les données ont été traitées à l'aide du logiciel R 4.1.1.

Des échantillons de sang ont été prélevés sur chaque femme lors du premier examen médical de grossesse et à l'accouchement. Pour le groupe test, nous avons utilisé une collection de sérums de l'Hôpital Mère-Enfant du CHU Dupuytren (biobanque CRBioLim certifiée NFS96-900). Pour le groupe témoin, des échantillons de sang frais ont été utilisés.

Pour déterminer les anticorps IgM et IgG anti-HCMV spécifiques, un analyseur de chimiluminescence automatisé LIAISON XL a été utilisé. Tous les patients ont également été testés pour le VIH et les virus de l'hépatite B et C. Aucun VIH, ni le patient infecté par l'hépatite n'a été inclus dans l'étude.

Des échantillons placentaires ont été obtenus pour le groupe test de la collection de pathologie du CHU Dupuytren (2016-2018) – échantillons congelés conservés à -80*C. Pour le groupe témoin, j'ai suivi strictement le protocole de prélèvement du service de Pathologie. Ainsi, le placenta frais entier a été prélevé après la naissance et conservé à 4*C avant traitement (au plus tard 72 heures). Une biopsie "carotte" pleine épaisseur a été prélevée et immergée dans de l'azote liquide pendant 5 secondes, puis immédiatement transférée sur de la neige carbonique, et puis stockée dans les plus brefs délais à -80*C.

Expression de gènes impliqués dans l'athérosclérose

Des échantillons placentaires ont été utilisés pour analyser et comparer l'expression de gènes spécifiques de l'athérosclérose. Ainsi, 4 groupes différents ont été conçus, chacun d'eux contenant trois échantillons :

- Grossesse normale HCMV négatif
- Grossesse normale HCMV positif
- PE HCMV négatif
- PE HCMV positif

Chaque échantillon congelé a été sectionné avec un cryotome à cinq niveaux différents. 20 lames de 10 µm chacune ont ensuite été stockées dans 1 ml de trizole, TRI Reagent® RNA Isolation Reagent pour réaliser l'extraction de l'ARN. Après 5 minutes de dissociation, 0,2 ml de chloroforme froid (-20°C) a été ajouté. Les tubes ont été vigoureusement agités à la main pendant 15 secondes puis laissés à température ambiante pendant 2-3 minutes. Ensuite, une centrifugation a été effectuée à 11200 tr/min, à 4*C, pendant 15 minutes. Ensuite, le surnageant a été transféré dans un nouveau tube. 600 ul d'éthanol à 70 % à température ambiante ont été ajoutés et mélangés par pipetage. Ensuite, le mélange a été transféré dans une colonne de travail et l'extraction s'est poursuivie selon le protocole standard du kit QIAmp® RNA Blood Mini. À la fin de l'extraction, chaque échantillon a été testé pour la qualité de l'ARN à l'aide des mesures du spectrophotomètre NanoDrop et de l'électrophorèse de l'ARN à l'aide d'un kit standard Agilent® RNA 6000 Nano. Les échantillons utilisés pour l'identification de l'expression génique avaient une bonne qualité d'ARN similaire. De plus, l'ADNc a été synthétisé à l'aide du kit Qiagen RT2 First Strand®. L'ADNc a ensuite été amplifié avec le RT² Profiler PCR Array en temps réel

(QIAGEN, Cat. No. PAHS-038Z) en combinaison avec RT² SYBR® Green qPCR Mastermix (Cat. No. 330529). Les valeurs Ct ont été exportées vers un fichier Excel pour créer un tableau des valeurs Ct. Ce tableau a ensuite été téléchargé sur le portail Web d'analyse des données à l'adresse http://www.qiagen.com/geneglobe. Le rapport d'analyse des données a été exporté à partir du portail Web QIAGEN sur GeneGlobe.

Pour évaluer l'expression des gènes, nous avons comparé les groupes d'échantillons suivants :

1. **Placenta normal HCMV POSITIF** (groupe test) à **placenta normal HCMV NÉGATIF** (groupe témoin)
2. **Placenta POSITIF HCMV PE** (groupe test) à **placenta NÉGATIF HCMV PE** (groupe témoin)
3. Placenta **POSITIF HCMV PE** (groupe test) à Placenta **normal POSITIF HCMV** (groupe témoin)

Analyse histopathologique

Des coupes congelées de 5 µm de biopsies placentaires ont été réalisées à l'aide d'un cryotome. Ils ont été fixés dans une solution froide d'acétone pure (90%) pendant 20 minutes. Une coloration à l'hématoxyline-éosine-safran (HES) a été réalisée automatiquement afin de rechercher le décollement de l'intima artérielle. Les sections colorées ont été analysées avec un scanner de diapositives numérique, Hamamatsu® NanoZoomer et le logiciel de visualisation NDP.

Une immunomarquage a également été réalisée afin de rechercher la présence de HCMV dans les vaisseaux sanguins placentaires. Plusieurs marqueurs ont été ciblés avec un protocole d'immunofluorescence. Les cibles étaient les cellules endothéliales (CD31), les cellules musculaires lisses (muscle lisse alpha actine), les macrophages (CD 68), les antigènes HCMV IE et le DAPI. Les anticorps utilisés pour la technique d'immunofluorescence et les dilutions sont présentés dans le tableau 1.

Tableau 1. Anticorps et dilutions utilisées pour l'immunocoloration

Cible	Anticorps primaires	Dilution	Anticorps secondaires	Dilution
Cellules endotheliales	-	-	**Ozyme SigmaAldrich** BLE303126, Alexa Fluor 594 anti-human CD31	1/50
Macrophages	-	-	**Abcam**	1/50

			Recombinant Alexa Fluor® 647 Anti-CD68 antibody [EPR20545] (ab224029)	
Cellules musculaires lisses	-	-	**Termo-Fischer** 25UG AntiAlpha-Smooth Muscle Actin eFluor 570	1/1500
Antigen HCMV IE	**Abcam** Anti-CMV IE1 and IE2 antibody [CH160] (ab53495)	1/50	**Abcam** Goat Anti-Mouse IgG Alexa Fluor® 488 (ab150113)	1/200
Nucleus	Abcam DAPI Staining Solution (ab228549)	1/1000	-	

Les sections congelées fixées ont été lavées avec une solution de PBS 3 fois pendant 5 minutes. La perméabilisation cellulaire a été effectuée avec du PBS-Triton 0,1 % pendant 5 minutes à température ambiante. Un autre lavage a été effectué avec du PBS 3 fois pendant 5 minutes. Ensuite, les sites non spécifiques ont été fixés avec du PBS-BSA 0,5 % pendant 30 minutes à température ambiante. 50 ul d'anticorps anti-antigènes IE HCMV de souris non conjugués (dilution 1/50) ont été ajoutés aux coupes, suivis d'une incubation pendant la nuit à 4 degrés. Le lendemain, un lavage a été effectué à l'aide d'une plateforme d'agitation - 3 fois pendant 10 minutes. Ensuite, 1500 µl de mélange d'anticorps conjugués anti-CD31, anti-CD68, anti-SMC et anti-HCMV IEA (chèvre anti-souris) ont été obtenus, en respectant les dilutions mentionnées ci-dessus pour chaque anticorps spécifique. Ce mélange a ensuite été ajouté aux sections et incubé pendant une heure à 37 degrés. Après cela, un nouveau lavage -3 fois x 10 minutes a été effectué. L'étape suivante était la coloration DAPI avec une dilution de solution DAPI 1/1000 pendant 5 minutes dans un endroit sombre, à température ambiante. Le dernier lavage - 3 fois x 5 minutes a été effectué en utilisant une solution PBS. À la fin, les sections ont été fixées avec un liquide spécial et vernies. Avant l'analyse au microscope confocal, toutes les coupes ont été stockées à l'abri de la lumière à 4 degrés. Pour l'analyse, un microscope confocal Carl Zeiss

Zen2 a été utilisé. L'acquisition et le traitement des images ont été réalisés avec le logiciel ImageJ, sur la plateforme de microscopie confocale (plateforme Biscem Inserm, Université de Limoges).

RÉSULTATS

La séroprévalence de l'infection latente à HCMV, mise en évidence par la présence d'anticorps IgG spécifiques anti-HCMV, était comparable dans la population générale et chez les patients VIH positifs. Il n'y avait pas de différences statistiques, entre les deux groupes, 92,7% chez les patients séropositifs vs 92,9% chez les patients séronégatifs, p-value de 0,93. Nous avons comparé nos résultats à ceux de la France et des territoires français. Il y avait des différences significatives dans la séroprévalence globale, 55,3 % en France contre 92 %. Fait intéressant, dans les deux pays, l'âge de l'infection est très bas, en dessous de l'âge de 5 ans. Dans le groupe VIH positif, les sujets avec des titres plus élevés d'anticorps IgG HCMV (> 8,76 UI/ml) avaient des valeurs significativement plus faibles du nombre actuel de cellules CD4 (367 vs 523, p = 0,02), du rapport CD4/CD8 (0,39 vs 0,74, p = 0,01) et du nombre de CD4 nadir (45 vs 143, p = 0,003), ainsi que des valeurs significativement plus élevées d'ARN VIH zénith (5,3 vs 4,2 copies/ml, p = 0,001) (figure dix). Il n'y avait pas de corrélation significative entre les paramètres de risque cardiovasculaire (triglycérides sériques, cholestérol, glycémie, valeurs de tension artérielle) et le taux d'anticorps anti-CMV. Malheureusement, l'évaluation de la fonction ventriculaire gauche ou l'évaluation de l'épaisseur de l'intima-média n'était pas disponible pour évaluer un risque accru précoce d'athérosclérose. Une étude prospective recherchant ces aspects apportera des informations supplémentaires importantes sur le risque clinique de maladies cardiovasculaires chez ce groupe vulnérable de patients. Nous supposons qu'une infection à CMV précoce, avec de multiples réactivations possibles au cours de la vie, déclenche un processus inflammatoire chronique qui est amplifié chez les patients présentant des facteurs de risque associés (obésité, diabète, tabagisme), augmentant la sévérité des maladies cardiovasculaires en Roumanie. Pour évaluer davantage cette hypothèse, nous avons étudié une éventuelle corrélation entre les titres d'anticorps anti-HCMV, en tant qu'expression de réactivations virales multiples et le risque cardiovasculaire dans un groupe de sujets immunocompétents, hospitalisés pour diverses raisons. Des titres plus élevés d'anticorps IgG anti-HCMV étaient associés à des modifications cardiaques plus importantes mais n'étaient pas corrélés à une augmentation de la masse ventriculaire gauche. Cependant, la petite dimension de notre cohorte, ainsi que le caractère unicentrique de notre travail, sont des biais qui doivent être corrigés dans des études

ultérieures pour une meilleure vision du rôle de l'infection à HCMV dans les maladies cardiovasculaires dans un pays à très forte prévalence de cette infection qui a en plus l'un des taux de mortalité due aux pathologies cardiovasculaires les plus élevés d'Europe. Dans le groupe séropositif, les sujets avec des titres plus élevés d'anticorps IgG HCMV étaient ceux qui avaient une immunosuppression plus sévère et des charges virales plus élevées au cours de leur maladie (comme le montrent les valeurs plus élevées du nadir CD 4 et du zénith VL), ce qui les rendait sujets à réactivations plus fréquentes du CMV. Pour l'instant, ces sujets sont encore très jeunes et ne présentent pas de corrélation significative entre les paramètres de risque cardiovasculaire (valeurs de triglycérides sériques, de cholestérol, de glycémie et de tension artérielle) et le taux d'anticorps anti-CMV. Malheureusement, l'évaluation de l'épaisseur de l'intima-média n'était pas disponible pour évaluer un risque accru précoce d'athérosclérose, et c'est un objectif futur important à atteindre.

Influences du cytomégalovirus humain dans la prééclampsie

Outre son rôle possible dans les maladies cardiovasculaires, le HCMV est la principale cause de troubles neurosensoriels chez les nouveau-nés et les enfants lorsque l'infection est acquise ou réactivée pendant la grossesse [28,32]. Dans le placenta, le HCMV était associé à une atteinte vasculaire [126] et au développement possible de troubles hypertensifs de la grossesse [38, 164]. La PE est une condition de la grossesse qui a été incriminée comme facteur de risque possible d'atteinte cardiovasculaire plus tard dans la vie, tant chez la mère que chez l'enfant [4,12]. Avec ces données de base, nous avons entrepris une nouvelle direction de recherche, visant à évaluer l'action dynamique du HCMV sur la paroi vasculaire, dans des groupes à risque particuliers, tels que les patientes ayant développé une prééclampsie pendant la grossesse. Avec l'opportunité d'une bourse de recherche internationale, nous avons pu évaluer le rôle de l'infection par le HCMV chez les femmes ayant des grossesses normales et une prééclampsie. Ainsi, à l'Université de Limoges, en France, nous avons conçu une étude qui a utilisé l'une des plus grandes cohortes de la littérature, à notre connaissance, d'échantillons placentaires de PE pour rechercher le rôle du HCMV dans le remodelage vasculaire et les lésions d'athérose aiguë. Notre première observation était que l'action cumulée d'une infection latente par le HCMV, évaluée par la présence d'anticorps IgG anti-HCMV, et un IMC > 25 kg/2 ou l'âge maternel influence la sévérité du PE. Ensuite, le statut sérologique HCMV positif de la mère influençait fortement le faible poids à la naissance. Avec l'aimable autorisation du service d'Anatomopathologie du CHU de la Mère et de l'enfant de Limoges, nous avons analysé une collection de 72 prélèvements placentaires congelés, 52 de grossesses PE et 20 de

grossesses normales. Pour le groupe test, sur 52 patients inclus dans cette étude, 33 (63%) avaient une infection latente à HCMV. Il n'y avait pas de différences statistiquement significatives entre les femmes EP HCMV positives et les femmes négatives en termes d'apparition précoce d'incidence de prééclampsie sévère, ni en présence de facteurs de risque comme la fécondation in vitro, l'hypertension, la présence de diabète gestationnel, l'IMC> 25 kg/ m2, fumeur. Seules les grossesses multiples étaient statistiquement significatives dans le groupe HCMV positif par rapport au groupe négatif (valeur de p 0,001). Les résultats histologiques suggèrent une athérose aiguë dans certaines sections de patients obèses HCMV positifs atteints du PE. Cependant, l'interprétation de ces résultats était assez difficile en raison d'un manque d'expérience avec des échantillons placentaires congelés. Non seulement il n'y a pas tellement d'études publiées sur le remodelage vasculaire dans les placentas humains, mais la majorité a utilisé des blocs ou des coupes inclus dans la paraffine qui ont un régime différent de techniques de coloration et d'interprétation. 12 échantillons ont été sélectionnés dans la collection pour des tests génétiques en fonction de leur qualité et quantité d'ARN total ainsi que pour des caractéristiques cliniques similaires. Ainsi, nous avons pu générer 4 groupes différents composés chacun de 3 échantillons - 1 PE HCMV positif, 1 PE HCMV négatif, 1 grossesse normale HCMV positive et 1 grossesse normale HCMV négative. Nos premiers résultats sur l'expression des gènes de l'athérosclérose humaine, à l'aide d'un kit commercial, ont révélé certaines différences d'expression biologique et statistique entre les grossesses normales positives pour le HCMV et les grossesses normales négatives pour le HCMV, ainsi que des changements plus importants dans les échantillons positifs pour le PE HCMV par rapport aux échantillons normaux positifs pour le HCMV. Cependant, les changements dans l'expression des gènes entre les deux groupes étaient assez hétérogènes et ciblaient principalement les gènes qui codent pour les facteurs chimioattractants et les molécules d'adhésion.

Pour une meilleure approche, nous avons effectué une analyse transcriptomique pour les mêmes 12 échantillons qu'auparavant, en espérant une signature HCMV particulière. Les résultats de la transcriptomique ont montré une excellente qualité de l'ARN total de nos échantillons après 4 méthodes d'évaluation différentes, mais aucune séquence HCMV n'a été identifiée. Ceci était inattendu car nous avons inclus dans nos échantillons 2 PE avec une éventuelle réactivation virale (anticorps IgM anti-HCMV équivoques et positifs). Ces résultats ne doivent pas être interprétés comme une absence manifeste de HCMV. Premièrement, nous savons que la distribution du HCMV dans le placenta est inégale et

peut parfois se reposer uniquement au niveau des artères spiralées sans traverser toute la structure placentaire [126, 149]. Ainsi, les échantillons de biopsie que nous avons utilisés pourraient avoir manqué les points infectés. Deuxièmement, il n'y a aucune preuve certaine de réactivation virale dans nos échantillons, car aucune charge virale sanguine n'était disponible et parfois la présence d'anticorps IgM peut être due à une réactivité croisée. Cette analyse du transcriptome est extrêmement complexe et compliquée et nécessite un travail d'équipe, de l'expérience et du temps pour une bonne interprétation. Par conséquent, au moment de la rédaction de cet article, l'analyse de ces résultats est toujours en cours. Cependant, nos premières données suggèrent les changements génétiques les plus significatifs dans la PE par rapport aux grossesses normales et entre les échantillons normaux positifs pour le HCMV et les échantillons normaux négatifs pour le HCMV, ainsi qu'aucune différence significative entre les 2 groupes PE. Ces résultats préliminaires sont assez similaires à ceux observés avec l'expression génique évaluée à l'aide d'un kit commercial, et ils mettent également en évidence des gènes impliqués dans l'adhésion cellulaire, ainsi que certaines séries de gènes qui codent pour la réponse immunitaire. D'après ce que nous savons jusqu'à présent, il s'agit de la première étude à examiner le réseau vasculaire villeux et l'interface fœtale du placenta humain en relation avec le remodelage vasculaire et l'expression des gènes.

CONCLUSION

Nos données suggèrent une très forte séroprévalence du cytomégalovirus humain dans la population roumaine, supérieure à 90%, avec une acquisition très précoce de l'infection, avant l'âge de 5 ans. Dans notre étude, la sévérité de la maladie cardiovasculaire était corrélée à des titres plus élevés d'anticorps IgG anti-cytomégalovirus humain mais n'influençait pas la masse ventriculaire gauche. L'évaluation du profil du gène athérosclérotique montre des changements importants dans l'expression des gènes entre les échantillons positifs pour le cytomégalovirus humain prééclampsie et les échantillons positifs pour le cytomégalovirus humain normal, ainsi qu'entre les échantillons positifs pour le cytomégalovirus humain normal par rapport aux échantillons négatifs pour le cytomégalovirus humain normal une fois.

En corroborant toutes les données obtenues à la suite de ces travaux, nous pouvons affirmer que le cytomégalovirus humain est plutôt un cofacteur dans l'initiation et le développement des maladies cardiovasculaires. Cependant, pris ensemble, tous nos résultats suggèrent un rôle auxiliaire du HCMV dans le remodelage vasculaire. Par

exemple, il peut interférer avec le réseau vasculaire utérin conduisant à la PE, mais sans avoir une influence directe sur les résultats cardiovasculaires à long terme du nouveau-né ou de la mère. L'état prééclamptique lui-même peut avoir un impact direct sur les résultats cardiovasculaires à long terme par un mécanisme qui doit être clarifié. Espérons que nos résultats finaux d'analyse transcriptomique ajouteront quelques briques d'information supplémentaires dans cette direction.

D'autres études approfondies sur des cohortes plus importantes de patients devraient être réalisées afin de répondre à des questions plus complexes sur le rôle et le mécanisme d'action du cytomégalovirus humain dans la pathologie cardiovasculaire.

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