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Effects of EVs Isolated from preeclamptic women on podocytes and renal endothelial glomerular cells (GEC)

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INDEX

INDEX

SUMMARY 5
INTRODUCTION 9
1. SYMPTOMS OF PREECLAMPSIA.. 10
2. TYPES OF PREECLAMPSIA 11
3. RATIONALE OF DISEASE 11
3.1. *Inflammation* 12
3.2. *Endothelial Dysfunction* 12
3.3. *Oxidative stress* 14
4. RENAL GLOMERULUS ENDOTHELIAL CELLS (GEC).. 16
5. PHYSIOPATHOLOGY OF GLOMERULAR DYSFUNCTION.................................. 17
6. ROLE OF EXTRACELLULAR VESICLES 19
7. OBJECTIVE OF THESIS 22
MATERIALS AND METHODS 23
Patients and Healthy controls..23
Clinical Evaluation 24
Blood Sample collection 25
EVS Nanoparticles Tracking Analysis...25
MACS Plex Exosome Kit .... .25
Fluorescence activated Cell Sorting 25
IN VITRO EXPERIMENTS 26
Cell Culture 26
NO release on GEC. 26
Cell viability on GEC and Podocytes 26
ROS release on GEC and Podocytes 27
Albumin Diffusion across Podocytes and GEC monolayer...27
Quantification of VEGFA &Endothelin1 release by Podocytes &GEC............................... .27
Nephrin Expression by Podocytes.. .28
STASTICAL ANALYSIS 28
RESULTS 30
DISCUSSION…………...52
LIMITATIONS AND FUTURE PRESPECTIVES...57
BIBLIOGRAPHY 60
ACKNOWLEDGMENT 65

SUMMARY

RATIONALE OF THE STUDY

The rationale of the study is to investigate the effects of extracellular vesicles (EVs) isolated from the plasma of preeclamptic women on podocytes and renal endothelial glomerular cells (GEC). Preeclampsia is a pregnancy-related complication characterized by high blood pressure, proteinuria, and organ damage. The study aims to characterize the EVs isolated from the plasma of preeclamptic women at different times during pregnancy and up to one month after delivery, and to analyse their impact on podocytes and GEC. The comparison is made with EVs isolated from the plasma of non-preeclamptic pregnant women (healthy controls).

The rationale is based on the understanding of oxidative stress, nitrosative stress, and the role of extracellular vesicles in the pathophysiology of preeclampsia. The study was approved by the Ethical Committee-Maggiore Della Carità Hospital in Novara and adhered to Good Clinical Practice standards.

The study aims to contribute to the understanding of preeclampsia by providing insights into the pathophysiology of the condition and the potential role of EVs in mediating cellular responses. By characterizing the EVs isolated from the plasma of preeclamptic women and analysing their impact on podocytes and GEC, the study seeks to uncover the mechanisms underlying preeclampsia and its effects on renal function. Additionally, the comparison with EVs from non-preeclamptic pregnant women allows for a better understanding of the differences in EVs and their potential implications in the development and progression of preeclampsia. Overall, the study aims to advance the understanding of preeclampsia and potentially contribute to the development of diagnostic and therapeutic strategies for this condition.

PLANNING OF THE STUDY

The planning of the study involved the characterization of extracellular vesicles (EVs) isolated from the plasma of preeclamptic women at different times during pregnancy (Diagnosis, T0), delivery (T1) and up to one month after delivery (T2). The study aimed to analyse the effects of these EVs on podocytes and renal endothelial glomerular cells (GEC), with a comparison to EVs isolated from non-preeclamptic pregnant women (healthy controls at T1, T2). The study also involved the collection of demographic variables, gynaecologic anamnesis, data about delivery and newborns, as well as clinical evaluations, echocardiographic variables, and blood and urine sample collection from both preeclamptic patients and healthy controls.

Patients were enrolled from January 2021 to January 2022. The inclusion criteria for the study involved enrolling patients from the High-risk Obstetric Unit of the Gynaecology and Obstetrics Department of the Maggiore Della Carità University Hospital in Novara. For preeclamptic patients, the inclusion criteria were as follows: age over 18 years; gestational hypertension, preeclampsia, preeclampsia with chronic hypertension, HELLP syndrome, and eclampsia. These patients were further subdivided into

severe and mild preeclampsia based on specific clinical indicators such as blood pressure levels, thrombocytopenia, creatinine levels, plasma transaminases, respiratory distress, neurologic symptoms, and epigastric/abdominal pain. For healthy controls, the inclusion criteria were women over 18 years of age with the absence of hypertensive disorders during pregnancy. Compliance with follow-up was also considered as an inclusion criterion for both groups.

The isolation and analysis of EVs were conducted using Nano Sight equipped with Nanoparticle Tracking Analysis (NTA) & NTA Analytical Software. Moreover, MACSPLEX and FACS analysis were done to characterize EVs in terms of lymphocyte/inflammatory/endothelial origin. Additionally, *in vitro* experiments were performed on GEC and podocytes to measure various cellular responses, including NO and ROS release, cell viability, albumin diffusion, VEGF A and endothelin 1 release, and Nephrin expression. The overall aim was to provide insights into the pathophysiology of preeclampsia and the potential role of EVs in mediating cellular responses in this condition.

RESULTS

The findings of the study indicate notable differences in the isolation and characterization of EVs in preeclamptic women compared to healthy controls. The concentration of EVs was found to be higher in severe preeclampsia at certain timings compared to other timings and in comparison, to mild preeclampsia and healthy controls. This suggests a potential correlation between the severity of preeclampsia and the concentration of EVs. Additionally, the study compared the expression of lymphocyte/platelet/endothelial markers in EVs isolated from preeclamptic women at different stages of the disease and healthy controls.

The differences we found may reflect changes in the release and composition of EVs, potentially influencing their effects on cellular function and contributing to the development and progression of preeclampsia.

The implications of these differences are significant as they provide insights into the potential role of EVs in the pathophysiology of preeclampsia. The differential expression and characteristics of EVs in preeclamptic women compared to healthy controls indicate their potential involvement in immune response, inflammation, endothelial dysfunction, and placental function. Understanding these differences may enable the development of biomarkers to identify individuals at risk for developing preeclampsia and contribute to the development of diagnostic and therapeutic strategies for this condition.

The study also involved *in vitro* experiments to measure various cellular responses, in GEC and podocytes including cell viability, ROS release, albumin permeability, NO release, Nephrin release, VEGFA and endothelin 1 release. The results showed differences in cellular responses between preeclamptic patients and healthy controls, for all timings. Hence, we found a lower cell viability both in GEC and podocytes and lower endothelin-1 release in GEC and increased ROS release and albumin permeability in both GEC and podocytes and increased NO release in GEC, and increased VEGF A

release in podocytes. As, regarding the Nephrin release by podocytes, we found reduction at T0 severe preeclamptic patients than the healthy controls.

CONCLUSIONS

The results of this research can contribute to the understanding of the physiopathology of preeclampsia particularly as regarding the role of EVs in mediating cellular responses. The study involved the characterization of EVs isolated from the plasma of preeclamptic women at different times during pregnancy and up to one month after delivery, as well as the analysis of their impact on podocytes and GEC. The comparison with EVs from non-preeclamptic pregnant women allowed for a better understanding of the differences in EVs and their potential implications in the development and progression of preeclampsia. The study can potentially contribute to the development of diagnostic and therapeutic strategies for this condition. The study's detailed methods and comprehensive analysis shed light on the complex interplay between EVs and preeclampsia, offering valuable insights for further research and clinical applications.

INTRODUCTION

PREECLAMPSIA

INTRODUCTION

One of the earliest descriptions of pre-eclampsia was published in 1637 by Francois Mauriceau, an early pioneer of the specialty of obstetrics. He noted the high risk of seizures in pre-eclampsia as well as the increased risk of this condition in primigravidas. Mauriceau attributed the development of eclamptic seizures to either abnormal lochial blood flow or intrauterine fetal death. In the 1700s, Boissier de Sauvages theorized that eclamptic seizures were nature's attempt to rid itself of a "morbid element". He made the important distinction between epilepsy and eclampsia on the basis of the resolution of symptoms postpartum in the latter.[1]

Preeclampsia was further defined in 1843 by John Lever, who found that the urine of women with pre-eclampsia contained albumin, and by Robert Johns, who noted the characteristic symptoms of headache, vision changes and oedema in affected patients. In the 1960s, researchers discovered the involvement of impaired placental implantation in pre-eclampsia, and in 1989 Roberts et al. hypothesized that the impaired placental perfusion seen in pre-eclampsia led to widespread maternal endothelial dysfunction. [2]

Preeclampsia is a pregnancy-related complication characterized by high blood pressure (blood pressure greater than 140/90 mm Hg) and damage to organs, most commonly the liver and kidneys. In preeclampsia, formerly called Toxaemia, pregnant women have high blood pressure, protein in their urine (greater than 300 mg per day), swelling in their legs, feet & hands. It can range from mild to severe. It usually occurs after 20 weeks of pregnancy in women whose blood pressure had previously been in the standard range and affects about 5-8% of pregnancies worldwide. If left untreated, preeclampsia can lead to serious complications for both the mother and the baby, fetal growth restriction, and maternal organ damage.[3]

The exact cause of preeclampsia is not fully understood but it is believed to be related to problems with the blood vessels in the placenta Figure 1.[4]

Preeclampsia is more likely to occur in first-time pregnancies, women over the age of 35, women with a history of high blood pressure or kidney disease and in women carrying multiples. Preeclampsia treatment includes careful monitoring and manage complications. [5]

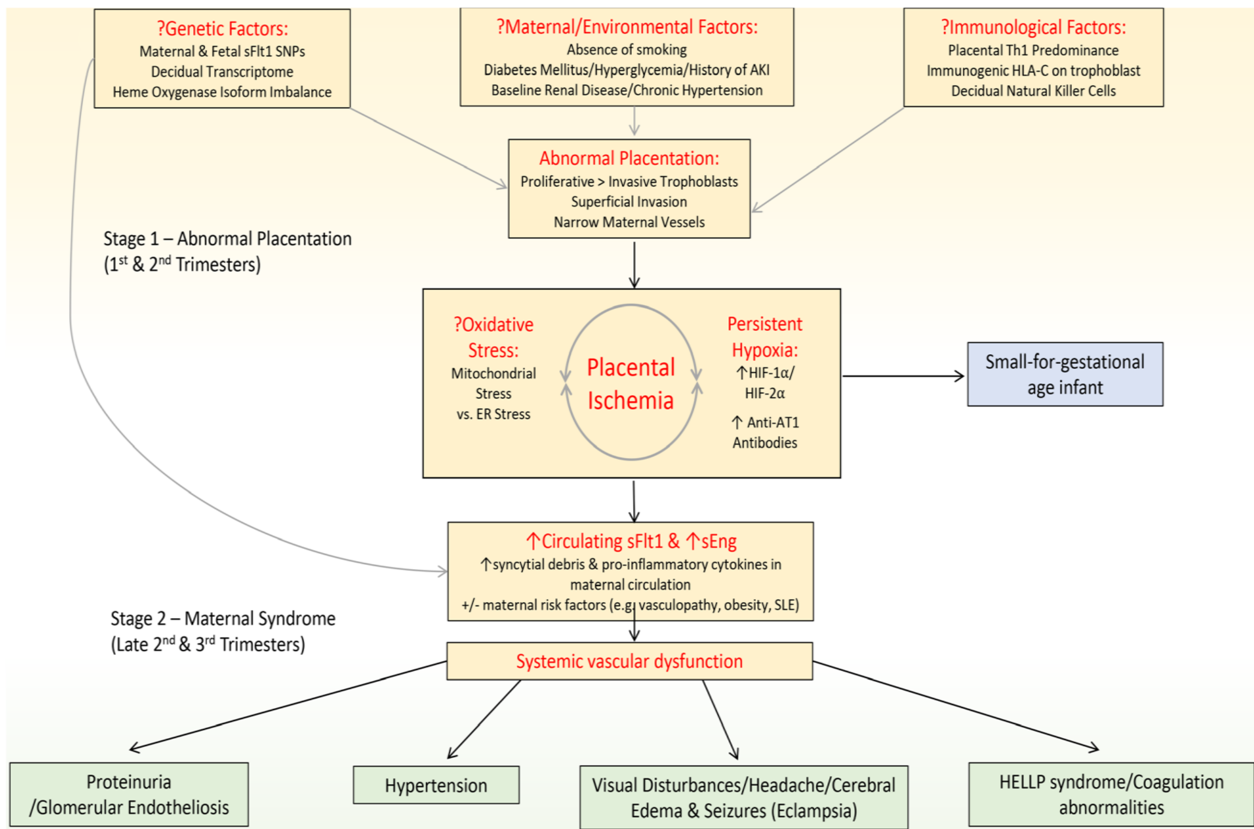


Figure 1: Schematic pathogenesis of preeclampsia. Genetic factors, immunologic factors, other maternal factors cause placental dysfunction which in turn leads to the release of antiangiogenic factors (such as sFLT1, soluble fms-like tyrosine kinase 1, and sENG, soluble endoglin) and other inflammatory mediators which can induce preeclampsia

1. SYMPTOMS OF PREECLAMPSIA:

- High blood pressure (hypertension)
- Protein in the urine (proteinuria)
- Swelling of the hands and face
- Severe headaches, changes in reflexes or mental state
- Blurred vision or sensitivity to light
- Upper abdominal pain, usually under the ribs on the right side
- Decreased urine output
- Trouble breathing
- Dizziness
- Weight gain over 1 or 2 days because of a large increase in body fluid.

Some women with preeclampsia may not experience any symptoms, which is why regular prenatal care and monitoring is so important. Preeclampsia can be diagnosed through regular prenatal check-ups, which include blood pressure measurements and urine tests. If preeclampsia is suspected, additional tests may be ordered to check for organ damage or fetal distress. Treatment depends on the severity of

the condition and the gestational age of the fetus. Mild cases may be managed with close monitoring, bed rest, and medication to lower blood pressure. [6]

Severe cases may require hospitalization and early delivery of the baby. To prevent preeclampsia, it's important for pregnant women to attend regular prenatal appointments, maintain a healthy weight, and manage any pre-existing medical conditions. If preeclampsia is detected and treated early, most women and babies will have good outcomes. [7]

2. TYPES OF PREECLAMPSIA

I. Mild preeclampsia: This type of preeclampsia is characterized by high blood pressure (greater than 140/90 mmHg) and proteinuria (greater than 300 mg per day), but no other signs or symptoms.

II. Severe preeclampsia: This type of preeclampsia is characterized by more severe symptoms, including high blood pressure (greater than 160/110 mmHg), proteinuria (greater than 5 g per day), headaches, visual disturbances, upper abdominal pain, and decreased urine output. Severe preeclampsia can lead to complications such as seizures (eclampsia), stroke, and organ damage.

III. Early-onset preeclampsia: This type of preeclampsia occurs before 34 weeks of pregnancy and is associated with a higher risk of complications such as fetal growth restriction, preterm delivery, and neonatal complications.

IV. Late-onset preeclampsia: This type of preeclampsia occurs after 34 weeks of pregnancy and is typically milder than early-onset preeclampsia.

V. Superimposed preeclampsia: This type of preeclampsia occurs in women who have pre-existing hypertension or kidney disease, and is characterized by the development of new-onset proteinuria and worsening hypertension during pregnancy. [8]

The classification of preeclampsia is based on the severity of symptoms, the gestational age at onset, and the presence of other risk factors such as pre-existing medical conditions. Early detection and management of preeclampsia are important to prevent complications for both the mother and the baby

3. RATIONALE OF DISEASE

While the exact cause of preeclampsia is not fully understood, several factors are believed to contribute to its development. Preeclampsia is a complex and multifactorial pregnancy-related

disorder that involves several biological processes, including endothelial dysfunction, oxidative stress, inflammation, and angiogenic imbalance. [9]

3.1 *Inflammation*

Inflammation plays a role in the development of preeclampsia. During pregnancy, there is a normal inflammatory response that helps the body to adapt to the changes associated with pregnancy. However, in preeclampsia, this inflammatory response becomes excessive and dysregulated, leading to tissue damage and dysfunction. [10]

Inflammasomes are high in molecular weight, multimeric, and self-organizing protein complexes of the innate immune system which do not only play a significant role in inflammatory response activation and the release of IL-1β and IL-18, but also function like a finely tuned alarm in cellular apoptosis regulation by triggering and enhancing systems in response to stress and/or cellular infections. Following the inflammasome signalling activation, inflammatory processes can potentially promote the development and secretion of proinflammatory cytokines including danger signalling and pyroptotic cell death, i.e., quick inflammation induced apoptosis. Differently from immunosuppression which occurs in normal pregnancy, preeclamptic pregnancy is characterized by excessive immune activation. [11]

Th1 cells, NK cells, and self-reactive B cells stimulate the inflammatory response through cytokines activity, which results in an inappropriate trophoblast invasion and impaired spiral artery remodelling in early pregnancy. Uteroplacental hypoperfusion is, therefore, the cause of placental ischemia, which triggers oxidative-inflammation cascade and increases production of antiangiogenic factors: soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng).[12]

Preeclampsia is characterized by excessive and progressively increased immune activation with a rise in proinflammatory cytokines and antiangiogenic factors both in the intrauterine environment and maternal endothelium, which is the cause of placental dysfunction and maternal systemic complications. [13]

3.2 *Endothelial Dysfunction*

Endothelial dysfunction is believed to play a central role in the pathogenesis of preeclampsia. The endothelium is a layer of cells that lines the blood vessels, and it plays a crucial role in regulating blood flow, blood pressure, and blood clotting. Endothelial dysfunction is a key feature of preeclampsia and is believed to be caused by an imbalance between vasodilators (substances that widen blood vessels) and vasoconstrictors (substances that narrow blood vessels), In preeclampsia, the endothelium becomes damaged and dysfunctional, leading to impaired blood flow, hypertension, and organ damage. [14]

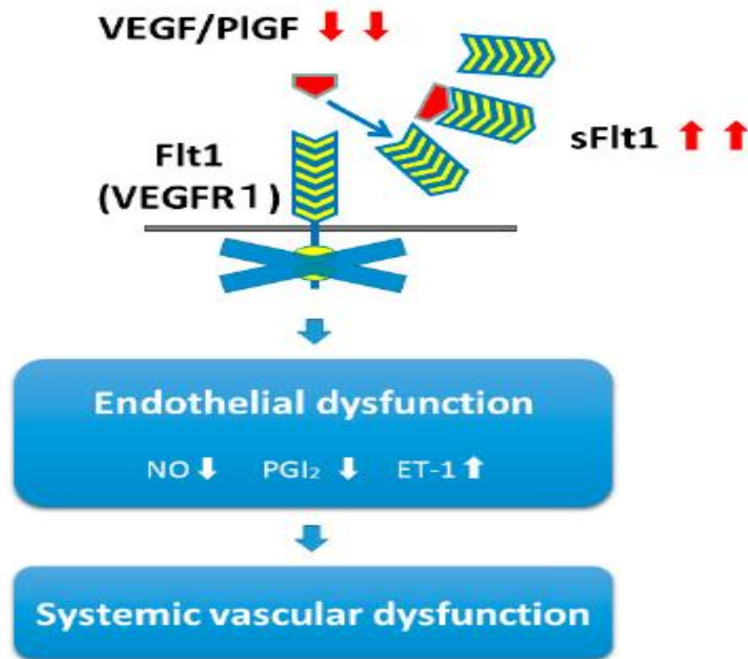
The molecular factors involved in preeclampsia are sEndoglin (sEngl) and soluble fms-like tyrosine kinase 1 (sFlt-1) or soluble vascular endothelial growth factor (VEGF) receptor, both affecting endothelial function (Figure 2). Membrane-bound Flt-1 is a receptor for VEGF, but sFlt-1 is a soluble, circulating splice isoform. Flt-1 and sFlt-1 mRNAs are upregulated in placenta of preeclamptic patients. No difference in expression is observed in the in vitro cell cultures from trophoblastic cells of normal or preeclamptic placentas. Upregulation is observed, however, in hypoxic conditions. Both VEGF and sFlt-1 are increased as gestation progresses, but are significantly elevated in women with preeclampsia. [15]

Thus sFlt1, an endogenous anti-angiogenic factor, act as a potent VEGF antagonist, is highly elevated in preeclampsia. VEGF is not only important in angiogenesis, but also important for maintaining endothelial health including the formation of endothelial fenestrae (a hallmark of the glomerular vascular endothelium). sFlt1 overexpression in animals induces glomerular endotheliosis with the loss of endothelial fenestrae that resembles the renal histological lesions of preeclampsia. [16]

More severe forms of preeclampsia, including the HELLP syndrome, may be explained by a concomitant elevation in both sFlt1 and sEndoglin, another anti-angiogenic factor, which is also elevated in sera of preeclamptic women. Soluble Endoglin is a soluble, placenta-derived, TGF-β co-receptor, and through inhibition of binding of TGF-β 1 to its receptor it might impair TGF-β signalling in the vasculature Figure 2. While VEGF, sEng, and sFlt-1 are mediators of endothelial dysfunction, other factors that lead to aberrant placentation, such as extracellular matrix proteins, hypoxia inducible factor 1 and 2, transforming growth factor beta family members and matrix metallo proteases, are also contributing to the syndrome. [17]

Other studies indicate that women with preeclampsia have auto-antibodies that activate the angiotensin receptor, AT1, and that autoantibody-mediated receptor activation contributes to pathophysiology associated with preeclampsia. Although these events are not directly linked to trophoblast invasion, there is evidence that preeclampsia may be a pregnancy-induced autoimmune disease.

Angiotensin is part of the rennin–angiotensin–aldosterone system, an important blood pressure regulation system. Renin is released into the blood by the kidneys, acts upon angiotensinogen, which undergoes proteolytic cleavage to form angiotensin I. Vascular endothelium, particularly in the lungs, produces the angiotensin converting enzyme (ACE), that cleaves angiotensin I to form the octapeptide, angiotensin II, which targets AT1. Activation of AT1, by angiotensin II or autoantibodies, triggers intracellular signalling pathways and mediates the major effects of vasoconstriction.[18]



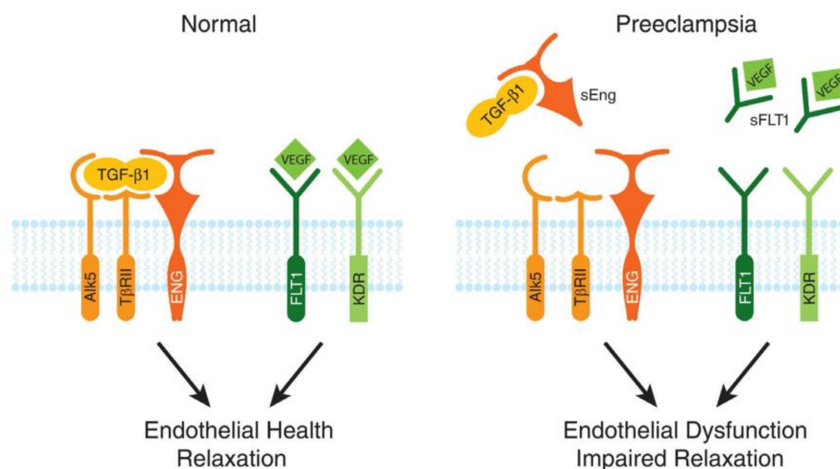


Figure 2: sFLT1 (soluble fms-like tyrosine kinase 1) and sENG (soluble endoglin) causes endothelial dysfunction by antagonizing VEGF (vascular endothelial growth factor) and TGF (transforming growth factor)-β1 signalling. There is mounting evidence that VEGF and TGF-β1 are required to maintain endothelial health in several tissues including the kidney and perhaps the placenta. During normal pregnancy, vascular homeostasis is maintained by physiological levels of VEGF and TGF-β1 signalling in the vasculature. In preeclampsia, excess placental secretion of sFLT1 and sENG (2 endogenous circulating antiangiogenic proteins) inhibits VEGF and TGF-β1 signalling respectively in the vasculature. This results in endothelial cell dysfunction, including decreased prostacyclin, nitric oxide production, and release of procoagulant proteins.

3.3 *Oxidative Stress in Preeclampsia*

Oxidative stress is another key factor in the development of preeclampsia. Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the body's ability

to detoxify them. ROS are highly reactive molecules that can damage cells and tissues, and they have been implicated in a variety of pregnancy-related disorders, including preeclampsia.[19]

Oxidative stress (OS) is defined as “an imbalance between oxidants and antioxidants, leading to a disruption of redox signalling and control and/or molecular damage”. OS involves ROS, the most common being superoxide ($O_2{\cdot}^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (•HO). A parallel process is known as nitrosative stress (RNS), which is defined as an imbalance in the ratio of nitrosants to antioxidants. NS principally involves the reactive nitrogen species (RNS) nitric oxide (•NO) and peroxynitrite ($ONOO^-$). In aerobic organisms, the controlled production of ROS and RNS is a physiological phenomenon that plays a fundamental role in metabolism and cell signalling cascades. [20]

OS and NS occurs when there is an imbalance between the formation of oxidizing substances and the antioxidant molecules that promote their detoxification. Because of their highly reactive properties, ROS and RNS can cause structural and physiological damage to DNA, RNA, proteins, and lipids, including cell membrane-bound lipids. Different cellular compartments or metabolic pathways can produce ROS and RNS. The mitochondria, endoplasmic reticulum (ER), and nuclear membrane produce O_2 anions due to the auto-oxidation of components of the electron transport chain (ETC). [21]

ROS are also produced as a consequence of arachidonic acid metabolism by Cyclooxygenase 2 (COX-2), Lipoxygenases, Xanthine Oxidase (XO), and Cytochrome P450. Nicotin Amide Dinucleotide Phosphate oxidases (NOX) are yet another important source of ROS. The NOX generate $O_2{\bullet}^-$ by transferring electrons from NADPH inside the cell across the membrane and reducing molecular oxygen. The NO synthase can generate $O_2{\bullet}^-$ and H_2O_2, in particular when concentrations of its substrate, L-arginine, or its cofactor, tetrahydrobiopterin (BH4), are low.[22]

Additionally, when intracellular ROS production increases (especially $O_2{\bullet}^-$), NO may react with ROS to form peroxinitrite, $ONOO^-$, a major cause of nitrosative stress ischemia/reperfusion episodes, thus creating a favourable environment for developing oxidative stress Figure 3. Oxidative damage in the placenta leads to inflammation, apoptosis, and the release of cellular debris into maternal circulation, along with several anti-angiogenic factors, such as sFlt1 and sEng, cytokines, and oxidants. These placental derived factors act on the maternal vascular endothelium, inducing OS and stimulating the production and secretion of pro-inflammatory cytokines, as well as vasoactive compounds. This results in a massive systemic endothelial dysfunction characterized by vascular inflammation and constriction. Indeed, OS appears to be the central component of both placental and endothelial dysfunction, the causative etiology of PE.[23]

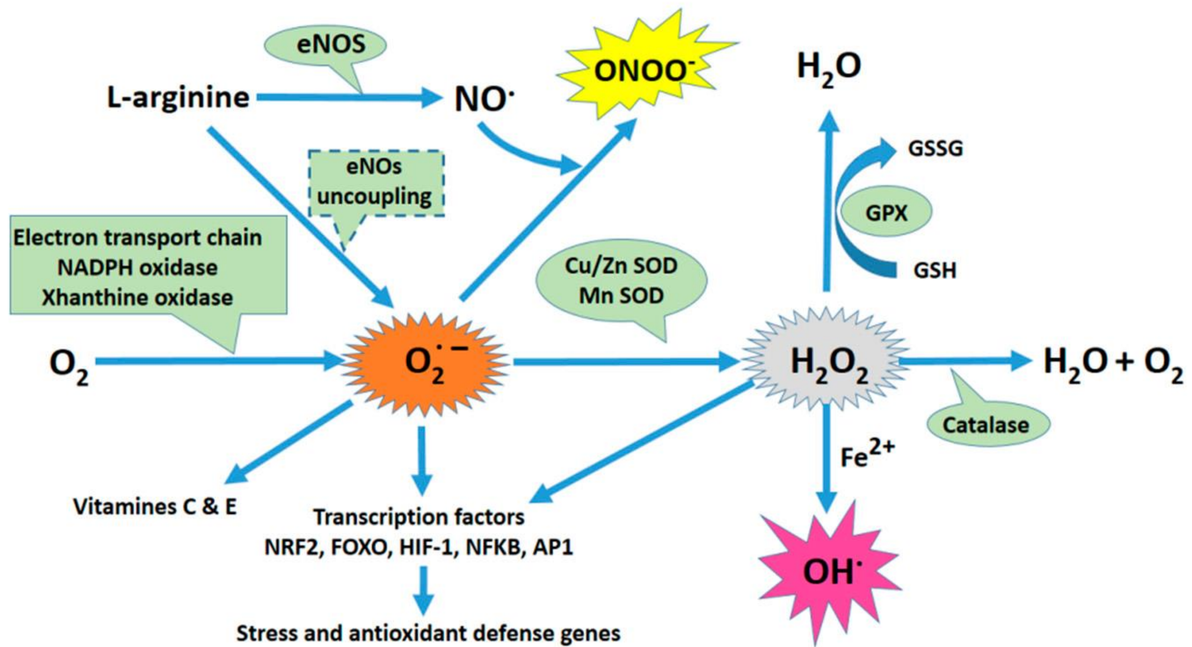


Figure 3. Oxidative stress plays a central role in the physiopathology of preeclampsia. Scheme of principal sources of oxidative stress and antioxidant mechanisms. The mitochondria, endoplasmic reticulum (ER), and nuclear membrane produce anions as a by-product of the auto-oxidation of electron transport chain (ETC) components. ROS are also produced from arachidonic acid.

Among its multiple effects on cell physiology, OS leads to the regulation of transcription factors such as AP-1, NRF2, Fox O, CREB, HSF1, HIF-1, TP53, NF-B, Notch, SP1, and CREB-1. Of these, only NRF2 and Fox O control the expression of several genes that encode enzymes required for the detoxification of oxidizing molecules.

The abnormal placenta of a PE patient is the source of ROS, as well as compounds that damage the endothelium, which itself becomes the source of ROS. Wang's experiment proved that OS causes damage to podocytes. The podocytes singled out from PE patient urine were shown to demonstrate no expression of nephrin, and none of the superoxide dismutase (SOD) that is normally present on the surface of the foot processes. The hypothesis says that in podocytes the role of SOD is to protect nephrin (its extracellular domain) against oxidative stress. [24]

4. RENAL ENDOTHELIAL GLOMERULAR CELLS (GEC)

The glomerulus is a highly specialized structure in which the capillary walls function as an efficient filtration barrier that restricts passage of larger molecules, predominantly proteins, but remains highly permeable to water and small molecules. These functions are achieved by the glomerular filtration barrier (GFB) comprised of an innermost fenestrated GEC layer, a glomerular basement membrane (GBM), and an outermost layer of podocytes with their interdigitating foot processes bridged by a slit

diaphragm. Injury of any of these three components of the GFB will lead to albuminuria and glomerular disease. [25]

GEC are one of the inherent cells of the glomerulus. As the first barrier of the glomerular filtration membrane, GEC are in direct contact with circulating substances in the blood and are more likely to be damaged by glucose, lipids and inflammatory factors. GEC play an important role in the occurrence and development of chronic kidney disease. The renal tubule interstitium accounts for more than 90% of renal parenchyma and performs a variety of functions. The surface of the renal tubule cavity is accompanied by a brush edge, which can increase the cell surface area and facilitate reabsorption in the renal tubule. In addition, GEC are metabolic cells that are rich in mitochondria, lysosomes and other organelles; they require a large amount of energy and are sensitive to damage.[26]

GEC have a unique feature of fenestrations and are able to handle a large amount of filtration. GEC injury is thought to contribute to the development of microalbuminuria, an early event of chronic kidney disease. However, despite their well-recognized association, the mechanism by which GEC dysfunction results in albuminuria is poorly understood. [27]

An extensive body of evidence indicates that proteinuria is often a result of ultrastructural changes in podocyte foot processes. However, many examples demonstrate that proteinuria may also occur independently of foot process effacement. For instance, mice with podocyte-specific overexpression of angiopoeitin-2 (Ang-2) had significant increases in both albuminuria and GEC apoptosis, while their podocytes remained structurally intact.[28]

5. PHYSIOPATHOLOGY OF GLOMERULAR DYSFUNCTION IN PREECLAMPSIA

Most researchers now agree that the glomerular filter cannot be regarded as individual layers but must be analysed as a whole. Several specific tasks can be attributed to the podocyte. First, podocytes synthesize GBM. This has been demonstrated in elegant studies by Abrahamson et al., who traced the origin of GBM components in equal amounts to endothelial cells and podocytes using a cell lineage tracing approach. Second, several groups showed in the 1960s that the podocyte is endocytically active. As theoretically any macromolecule may pass the glomerular filtration barrier, it can be assumed that podocytes remove at least some of the retentate from the outer GBM by endocytosis. [29]

Third, it is proposed that the complex cytoarchitecture of podocytes is optimized to facilitate generation of a filtration-dependent potential. As the glomerular filtration barrier has a very low electrical resistance, charged particles (i.e., ions) must be continuously separated across the entire filtering surface to generate the potential difference. The filters don’t allow proteins to cross it.
[30]

A potential difference (Gray arrows) is established across the filtration barrier by passage of the small ionic plasma components (i.e., dipole water, and small solutes sodium, chloride, etc.), which interact with the charged filter walls of the glomerular filtration barrier. As albumin and most plasma proteins are negatively charged.[31]

The podocyte cell bodies and primary processes cover about one half to two thirds of the filtering surface, and it has been proposed that podocyte cell bodies float within the primary urine only to enlarge filtration surface. However, filtration surface could be increased much easier by increasing the number of glomeruli. So why does nature bother to detach podocyte cell bodies? It was proposes that this is to reconcile two conflicting situations: Podocytes are necessary to synthesize and clear the GBM but at the same time present a potential obstacle for filtration and thus for homogeneous generation of a potential difference. [32]

To solve this problem, the podocytes cover the capillaries exclusively with interdigitating foot processes. This allows filtration to occur homogenously across the entire filtering surface so that a potential difference can be established homogenously and thus proteinuria be prevented. This notion is also consistent with the pathogenesis of proteinuria in minimal changes nephropathy, where podocyte foot processes are effaced. In summary, a model for glomerular filtration, which also considers electrical effects, provides a novel approach to our understanding of the function of the glomerular filter Figure 4. Podocytes play an intricate and essential part in this highly efficient biological system. Advances in our understanding of the glomerular filter will provide the premises to design novel therapeutical concepts.[33]

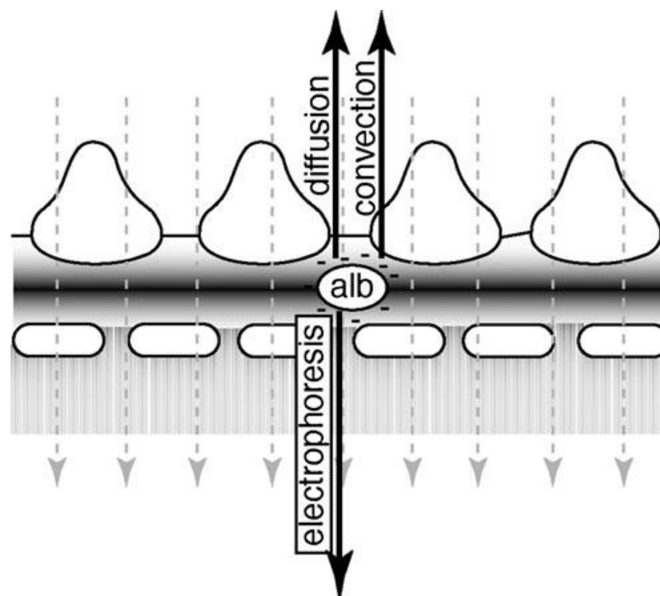


Figure 4: Glomerular filtration barrier. The filtrate passes the layers of the filter as a laminar, nonturbulent flow along an extracellular route (arrow). Albumin is largely excluded from entering the filter, as indicated by the local albumin concentration on the left. GBM Glomerular basement membrane

Proteinuria is the hallmark of pre-eclampsia that differentiates it from other hypertensive disorders of pregnancy, despite the controversy surrounding its usefulness in diagnosing pre-eclampsia. Proteinuria

is thought to be due to endothelial cell swelling and disruption of fenestrae. Over the last six years, most of the research has focused on derangements of podocytes and podocyte-specific proteins (such as nephrin, synaptopodin, podocin, and podocalyxin), and their roles in the mechanism(s) of proteinuria in pre-eclampsia. Studies of human tissue show that the expressions of podocyte-specific proteins are severely affected by pre-eclampsia. A study comparing renal sections from women with pre-eclampsia, compared to those from women with either normotensive or chronic hypertensive pregnancies, reported reduced expressions of podocyte-associated proteins, nephrin, glomerular epithelial protein 1, GLEPP-1, and ezrin in their renal tissue sections. [34]

Decreased glomerular expressions of nephrin and synaptopodin were seen in renal tissue sections from women who died from pre-eclampsia compared to those of women with normal pregnancies who died from other causes. Podocin expression, however, was relatively unchanged. The degree of podocyte dysfunction required for such dramatic changes in nephrin and synaptopodin expressions might be expected to cause changes in multiple other proteins important to the integrity of the glomerular filtration barrier and, possibly, podocyte attachment.[35]

The detection of podocyte products and live podocytes in the urine (podocyturia) suggests that podocyte pathology is more severe than might be inferred from renal biopsy studies. Various methods have been developed to detect urinary podocyte products. Culturing of urinary podocytes increases specificity by removing dead and non-specific cells, but is difficult and time consuming. Cytospin techniques, while quicker and possibly more amenable to automation, suffer from low sensitivity and specificity due to the large number of cellular debris. More sensitive techniques using reverse transcriptase-polymerase chain reaction (RT-PCR) and mass spectrometry remain in development. Number of podocytes positively correlates with the degree of proteinuria, suggesting a cause-effect relationship between ongoing podocyte loss and the onset and severity of proteinuria, that these are mechanistically related.[36]

The usefulness of podocyturia for early diagnosis of pre-eclampsia remains an active research topic. Other groups have confirmed that podocyturia is specific to the diagnosis of pre-eclampsia, using both podocalyxin and nephrin staining. A recent study using synaptopodin staining of urinary cytospins questioned the usefulness of this technique.

6. ROLE OF EXTRACELLULAR VESICLES IN PREECLAMPSIA PHYSIOPATHOLOGY

Microparticles, also named “macrovesicles” (MVs), Extracellular Vesicles (EVs) or Exosomes, are cell membrane surrounded vesicles that are formed during cell activation, apoptosis, and cellular stress. They directly bud into the extracellular space. and transmit the messages to adjacent and distant cells. Under both pathological and physiological conditions, different kinds of vesicles are produced. [37]

They are different from each other based on content, size, and generation mechanism. EVs are smaller than apoptotic bodies (800–5000 nm) but larger than exosomes (approximately 40–100 nm). EVs are

100–1000 nm globular-like structures, encapsulated by a plasma membrane that harbour's membrane lipids, anionic phospholipid, phosphatidyl serine (PS), and surface-specific membrane antigens that are very specific and indicate the components of the parent cell membranes. Figure 5. [38]

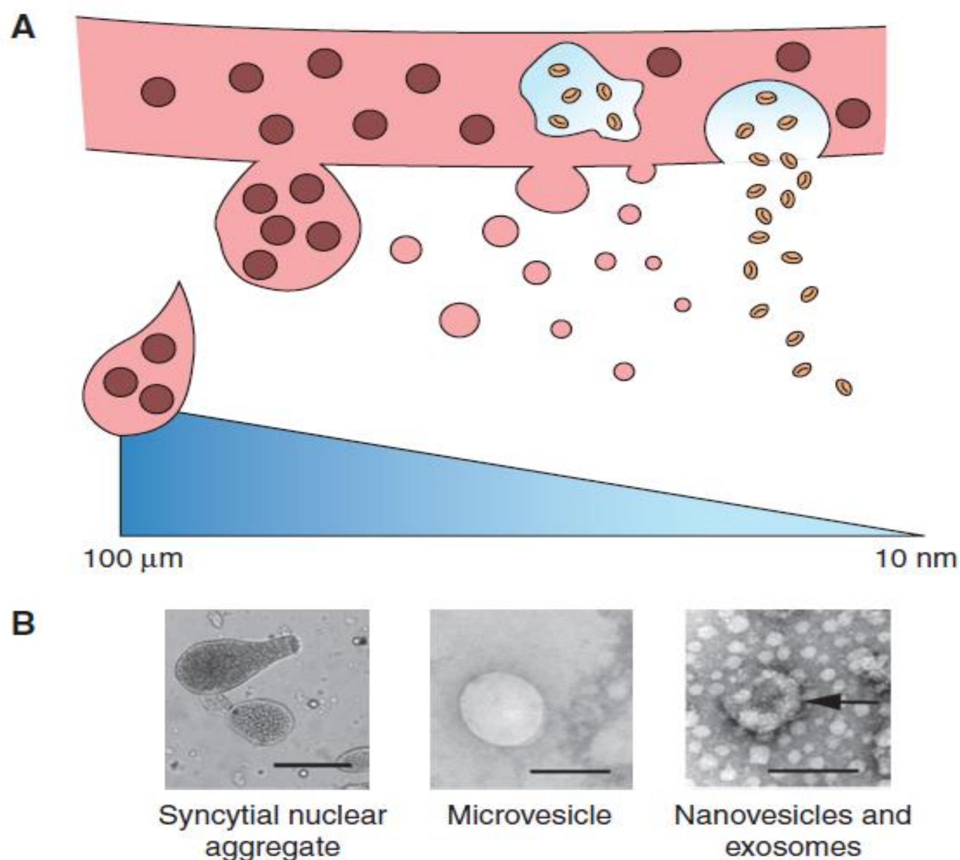


Figure 5: Different types of extracellular vesicles produced by the placenta. (A) A schematic representation showing the shedding of macrovesicles, microvesicles, and nanovesicles, including exosomes from the syncytiotrophoblast (Left to right). (B) Light micrograph of a syncytial nuclear aggregate (scale bar, 50 mm), transmission electron micrograph of a negatively stained microvesicle (scale bar, 100 nm), and transmission electron micrograph of a negatively stained exosome (arrow) with other nanovesicles (scale bar, 100 nm).

The cytoplasm of EVs contains different bioactive molecules including proteins, lipids, signalling molecules, mRNA, microRNA (miRNA), long non-coding RNAs (lncRNAs), and even DNA that regulate the cellular function, as paracrine or autocrine vehicles in the microenvironment. The MP-releasing process is started by various stimuli (e.g., inflammation) and mediated by a series of biomechanical and/or chemical factors. Most of the time, cytosolic calcium increases either by an extracellular inflow or an intracellular release is the initiator. As the calcium levels are elevated, the activity of Calpains and cytoskeletal rearrangements are started with the mediation of small GTPases. Simultaneously, the plasma membrane structure is altered and broken down by calcium-dependent activation of floppases, inactivation of flippases, and phosphatidylserine exposure. [39]
The results are vesicle forming, leading to external shedding of the plasma membrane. Because of their biogenesis, most of these vesicles carry membranous surface markers of their origin cell. It is believed that all cell types are capable to produce EVs and their products have been verified in vascular smooth

muscle cells, leukocytes, erythrocytes cardiomyocytes, podocytes, cancer cell, and many progenitor cells. Therefore, EVs can be found in vitreous body fluids, atherosclerotic plaques of the vascular wall, and extracellular spaces of solid organs. EVs mediate inflammation, thrombosis, and immune responses; thus, they are involved in diseases' physiological and pathological events. Another important feature of EVs is their heterogeneity; EVs that are released from the same cells could be different depending on the releasing stimuli. [40]

Additionally, different cell types with the same stimulus will release different EVs with different components. Microparticles are found in all biological fluids and plasma of healthy individuals, but their rate of production and probably contents are changed in various disease conditions. EVs (microparticles and exosomes) have been considered as biomarkers in many diseases such as lupus nephritis, diabetic nephropathy, pre-eclampsia, focal segmental glomerulosclerosis, and IgA nephropathy. As EVs are the fingerprint of their parent cells in a different pathophysiological situation, the importance of urinary derived-EVs came under the spotlight to illustrate valuable diagnostic.[41]

In general, microvesicles are derived from budding of the plasma membrane of cells however, the exact origin of placental microvesicles is unclear. The syncytiotrophoblast has a microvillous surface, which is important for slowing down the movement of maternal blood over the surface of the syncytiotrophoblast to facilitate increased transfer of nutrients and gases across the placenta [42]. It has long been thought that parts of the microvilli may be shed into the maternal blood as microvesicles and early preparations of microvesicles from the syncytiotrophoblast can involve mechanical disruption of the microvillous membrane.

Physiologically, what would cause the shedding of microvilli is unclear, as it was found that large amounts of microvesicles are shed from villous placental explants in static culture. A recent study reported that approximately one third of SNAs (Spherical Nucleic Acids) have obvious blebs on their surfaces, and this is a possible source of trophoblast microvesicles. The production of apoptotic blebs by the syncytiotrophoblast is another potential source of trophoblast microvesicles[43]. It is also possible that villous cytotrophoblasts exposed to the maternal blood following denudation of the syncytiotrophoblast or extra villous trophoblasts could also be minor sources of trophoblast microvesicles. Platelets and other cell types release microvesicles in response to activating stimuli, and it is possible that trophoblasts have a similar response to a variety of yet undetermined stimuli. The amount of trophoblast microvesicles in the maternal plasma has been reported to increase with increasing gestation in normal pregnancy, but this increase with gestation was not as marked in women with preeclampsia. EVs have a dynamic role in the communication among maternal vascular cells (the vascular endothelium, circulating leukocytes, and platelets) and the placenta, thus contributing to the progression of normal pregnancy.

Depending on Pre-existing maternal conditions, any of these vascular components during pregnancy may be capable of initiating the cascade of events that result in preeclampsia. In maternal conditions associated with the activation of vascular endothelial cells and immune system modulation, EVs can augment inflammation, coagulation, and endothelial dysfunction. Pre- pregnancy maternal platelet activation can augment endothelial dysfunction and inflammation via EVs, facilitating the progression to preeclampsia. In women without maternal risk factors associated with preeclampsia, it is possible

that placental trophoblast-derived EVs may contribute to the maternal milieu that favours progression to preeclampsia. Furthermore, understanding the roles of the specific types of EVs in the pathogenesis of preeclampsia may enable the development of a panel of biomarkers that will help to identify pregnant women at risk for developing preeclampsia.[44, 45] [46]

AIM

The aim of this study was to characterize the EVs isolated from plasma of preeclamptic women at different timings during pregnancy up to 1 month after delivery and to analyse their effects on podocytes and GEC. Comparison was done with EVs isolated from plasma of non-preeclamptic pregnant women (healthy controls).

MATERIALS AND METHODS

MATERIALS AND METHODS

Patients were enrolled from January 2021 to January 2022, at the High-risk Obstetric Unit of the Gynecology and Obstetrics Department of the Maggiore della Carità University Hospital in Novara. preeclamptic patients were eligible if matching the following inclusion criteria: age > 18 years; gestational hypertension, preeclamptic, preeclamptic+ chronic hypertension; HELLP; Eclampsia. Those patients were subdivided into severe and mild preeclampsia on the ground of the presence/absence of: systolic arterial blood pressure (SAP) > 160 mmHg or diastolic arterial blood pressure (DAP) >110 mmHg; thrombocytopenia (<100'000 PTL/microL); creatininemia > 1.1 mg/dl; doubling of plasma transaminases; respiratory distress or pulmonary edema; neurologic symptoms; epigastric/abdominal unjustified pain. preeclamptic patients were treated with hypertensive drugs (alfa-metildopa, nifedipine, labetalol; $MgSO_4$ in severe preeclampsia).

As concerning non-preeclamptic patients (healthy controls), inclusion criteria were: 18 years; absence of hypertensive disorders during pregnancy. Healthy controls were aged- and gestational aged-matched with preeclamptic patients.

In both cases, the absence of compliance to adhere to the follow up was considered an exclusion criteria.

In our hospital, which is a hub center for severe obstetric diseases, a total of 1850 deliveries occurred annually, with an average of 5% cases of PE. A total of 53 patients was recruited, among which 36 PE (18 severe and 18 mild) and 17 healthy controls.

The study was approved by Ethical Committee-Maggiore della Carità Hospital in Novara (Protocol n 26593, Study n CE172/17). Patients were explained the research protocol and study objectives, they were informed that they could live the study or be withdrawn at any time during the study for the onset of confounding factors and were asked to sign an informed written consent. Patients were treated according to the Good Clinical Practice standards.

1. CLINICAL EVALUTION

In all patients, demographic variables, gynecologic anamnesis, data about delivery and newborns were collected. In preeclamptic patients, systolic and diastolic blood pressure, plasma biochemical variables (transaminases, hemocrome) and renal function were examined at diagnosis (T0), delivery (T1) and at 40 days after delivery (1 month after delivery; T2). The same time-course was followed for the measurement of plasma EVs isolation. In healthy controls, the clinical evaluations were executed at T0 and T1, whereas plasma EVs isolation was performed at T2, too. In both preeclamptic patients and healthy controls, echocardiographic variables were measured at T1, as well.

I. *Collection of samples*

Blood samples were taken from preeclamptic patients (T0, T1 AND T2) and healthy controls (T1 and T2) by using BD Vacutainer tubes (sodium heparin as anticoagulant). Each sample was immediately centrifuged by a refrigeratedcentrifuge (Eppendorf, mod. 5702 with rotor A-4-38) for 10 min, at 3100 rpm at 4°C. The plasma obtained was divided into 5 tubes that were stored at -80°C at the Physiology laboratory of the University East Piedmont and used for the aspartate and alanine transaminases (AST, ALT), hemocrome, and uricemia analysis and EVs isolation.

II. *EVs nanoparticle tracking analysis*

Isolated EVs were diluted 1:1000 in a 0.22 μm filtered physiological solution (sodium chloride 0.9%; B. Braun; Milan, Italy) and analyzed by Nano Sight (NS300; Malvern Panalytical; Malvern, UK) equipped with the Nanoparticle Tracking Analysis (NTA) & NTA Analytical Software. The number of total EVs for each patient was obtained by multiplying the value given by the instrument (microparticles/milliliter) for the dilution made for the analysis and for the number of ml in which EVs were resuspended.

III. *MACSPlex Exosome Kit*

For this analysis, $1x10^{10}$ EVs was resuspended in the MACSPlex Buffer (Miltenyi Biotec S.r.l., Bologna, Italy) with 15 μL of the antibody-coated MACSPlex Exosome Capture Beads and 15 μL of MACSPlex Exosome Detection Reagent cocktail overnight at 4 °C under gentle agitation and protected from light. Thereafter, EV-bead complexes were washed using 500 μL MACSPlex buffer and centrifuged at 3000 g for 5 min at RT. The supernatant was removed and this step wad repeated two times and then, each sample was analyzed in triplicate with Attune™ NxT flow cytometer (Thermo Fisher Scientific). Background values of PBS and the isotype controls (REA or mouse IgG) were subtracted from each of the sample PE median fluorescence intensity value (MFI), resulting in "background corrected CD9/CD63/CD81/CD105 PE MFI". Background corrected CD9/CD63/CD81/CD105 MFI values were normalized to the mean signal of MFI for the CD9/CD63/CD81/CD105 beads within each sample resulting in background corrected CD9/CD63/CD81/CD105 normalized MFI values.[47]

IV. *Fluorescence-activated cell sorting (FACS)*

Attune™ NxT flow cytometer (Thermo Fisher Scientific) was used for the analysis of Tissue factor (TF; Thermo Fisher Scientific), CD42b, placental alkaline phosphatase (PLAP; Santa Cruz Biotechnologies,Dallas, Texas, USA) and CD178 (Thermo Fisher Scientific) in isolated EVs.
Briefly, EVs were diluted 1:100 with saline filtered with a 0.22 μm filter and dispensed into a 96-well plate. Subsequently, the antibody labeled with a fluorescent molecule was added in a 1:1 ratio

with the EVs. Thereafter, the plate was incubated for 1 hour at 4°C protected from light. The EVs from each patient and healthy controls were analyzed in duplicate.

The antibodies used were conjugated to the fluorescent molecule, fluorescein isothiocyanate (FITC) or phycoerythrin (PE). In particular, we used FITC mouse anti human CD42b, PE Mouse Anti-Human CD178, FITC Mouse Anti- Human PLAP and FITC mouse anti human TF. As a control, the EVs were also incubated with a FITC mouse IgG isotype control or PE mouse IgG isotype control (Thermo Fisher, Scientific).

2. *In vitro* experiments

I. *Cell culture*

Human GEC were gently given by Laboratory of Experimental Nephrology of Turin University, while, human primary podocytes were obtained from normal cortex fragments of surgically removed kidneys and immortalized by infection with a hybrid Adeno5/SV40virus, as previously described.
Each cell type was maintained in Dulbecco's Modified Eagle Medium (DMEM, Sigma) supplemented with 10% Fetal Bovine Serum (FBS, Euro clone) at 37°C with 5% CO_2 in incubator. We selected EVs isolated from 10 severe and 10 mild preeclamptic patients and from 10 healthy controls to perform the in vitro experiments on GEC and podocytes. Experiments on NO and ROS release, cell viability, albumin diffusion, VEGF and endothelin 1 release and Nephrin expression were conducted in duplicate or triplicate and repeated at least three times.

II. *NO release on GEC*

NO production was measured in GEC supernatants by using the Griess method (Promega, Milan, Italy), as previously performed in the same or similar cellular models.[48] GEC were stimulated with 50 000 EVs for each target cell for 12 h while, non-stimulated cells, were used as control. At the end of the stimulations, NO production in the sample's supernatants was examined by adding an equal volume of Griess reagent following the manufacturer's instruction. NO production was measured in the sample's supernatants at 570 nm (VICTOR™ X Multilabel Plate Reader; PerkinElmer; Waltham, Massachusetts, USA) and compared with values obtained from a standard curve, in order to quantify the NO production, which was expressed as nitrites (μM).

III. *Cell viability on GEC and podocytes*

Cell viability was examined in GEC and podocytes by using the 1% 3-[4,5-dimethylthiazol-2-yl]-

2,5-diphenyl tetrazolium bromide (MTT; Life Technologies Italia, Monza, Italy) dye, as previously described and following the same protocol described for Griess assay. The 10% of the MTT solution, was prepared dissolving 50 mg of the MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide) in 10 mL of PBS (pH 7.4) and kept stored at 4°C, protected from the light. After the treatment, the medium was removed, and 0,5 mg/mL of MTT dye was added to eachwell and incubated for 2 h at 37°C. Once the reaction has occurred, the supernatant was removed andthe formazan crystals formed in each well were dissolved with 100 µL of dimethyl sulfoxide (Sigma, Milan, Italy). Cell viability was determined by measuring the absorbance through a spectrometer (VICTOR™ X Multilabel Plate Reader) with a wavelength of 570 nm and cell viability was calculated by setting control cells (non –treated cells) as 100%.[49, 50]

IV. *ROS release on GEC and podocytes*

The oxidation of 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA) into 2,7-dichlorodihydrofluorescein (DCF) was used to assess ROS generation, following the manufacturer's instructions (DCFDA Cellular ROS Detection assay, Abcam, Cambridge, United Kingdom), as previously performed and following the same experimental protocol described for Griess and MTT assays. Briefly, after treatments, the medium was removed and 10 µM H2DCFDA was added for 20 min at 37°C. The fluorescence intensity of DCF was measured at an excitation and emission wavelength of 485 nm and 530 nm, respectively (VICTOR™ X Multilabel Plate Reader).[51, 52]

V. *Albumin diffusion across podocyte and GEC monolayer*

The percentage of albumin diffusion was evaluated by a colorimetric assay, as previously performed. Briefly, podocytes (500 000 cells/well) and GEC (300 000 cells/well) were grown in a complete medium on 0,4 µm pore size Trans well inserts in a 24-well culture plate (Life Science; Gerenzano, Milan, Italy) to form a confluent monolayer. Next day, at confluence, the medium was removed and the monolayer was covered immediately with 500 µL DMEM (FBS and phenol red free) containing 5% albumin, FITC conjugated and then, 50 000 EVs/target cells were added and incubated for 12 h. At the end of stimulations, albumin-FITC diffusion across the monolayer was quantified by measuring absorbance at 590 nm (VICTOR™ X Multilabel Plate Reader). The albumin-FITC diffusion was compared with control cells (non-treated cells) and expressed as:

$$\text{\% of diffusion} = \frac{\text{mean Abs}_{590\,nm}\text{ sample - mean Abs}_{590\,nm}\text{ control}}{\text{mean Abs}_{590\,nm}\text{ control}} \times 100$$

VI. Quantification of VEGF-A and endothelin-1 release by podocytes and GEC

For the experiments, podocytes and GEC were stimulated as described for Griess, MTT and ROS methods. VEGF-A and endothelin-1 release from podocytes and GEC, respectively, were detected by means of specific ELISA assay (R&D Systems, Inc.; Minneapolis, Minnesota, USA) through a spectrophotometer (VICTOR™ X Multilabel Plate Reader), at 540 nm.
In case of VEGF-A, after addition of 200 µL of standard, control, and supernatant sample per well, the plate was incubated for 2 h at room temperature. After washing with Wash Buffer (400 µL), 200 µL of Human VEGF Conjugate was added to each well and left in incubation for 2 h at room temperature. After washing again, 200 µL of Substrate Solution was added to each well, protected from light and left in incubation for 20 min at room temperature. Finally, 50 µL of Stop Solution was added to each well and the reading was executed as above described.[53]

In case of endothelin-1, 75 µL of standard, control, and supernatant sample wad added per well and incubation was executed for 1 h at room temperature. After washing with Wash Buffer (400 µL), 200 µL of Endothelin-1 Conjugate was added to each well and left in incubation for 3 h at room temperature. After washing again, 200 µL of Substrate Solution was added to each well and left in incubation for 30 min at room temperature, protected from light. Finally, 50 µL of Stop Solution was added to each well and the reading was executed as above described. The measurements were performed at least in triplicate.

VII. Nephrin expression by podocytes

For the experiments, 300 000 podocytes/well in 6-well plates were plated and stimulated with EVs, as described for Griess, MTT and ROS assays. After treatments, the detached cells were resuspended with 100 µL of filtered physiological solution to make a final concentration of $1x10^6$ cells/mL. Thereafter, 10 µg/mL of the antibody Anti-Human Nephrin, FITC conjugate (Santa Cruz Biotechnology; Dallas, Texas, USA) was added and incubated for 1 h at 4°C in the dark. The analysis was performed by using Attune™ NxT flow cytometer (Thermo Fisher Scientific).

3. Statistical analysis

All data were recorded using the Institution's database. Statistical analysis was performed by using GraphPad Prism 6 (San Diego, USA) for Microsoft Windows. As regarding categorical variables, data were checked for normality before statistical analysis. All the results obtained were examined through Mann Whitney. All data are presented as means ± standard deviation (SD), of repeated measurements. A value of $P <0.05$ was considered statisticallysignificant.

RESULTS

RESULTS

As a first step, we performed the **EVs characterization** for patients and healthy controls, and the results are illustrated in **Figures 6 and 7.**

EVs concentration of preeclamptic patients is higher than that of healthy controls at T1.

As shown in **Figure 6**, EVs concentration is higher in **severe preeclamptic patients at T0** as compared to **severe T2.** Also, EVs concentration is higher in **mild preeclamptic patients at T0** as compared to **mild T1, T2.**

Finally, the comparison between severe and mild preeclamptic patients shows that EVs concentration is higher in severe patients at all timings**.**

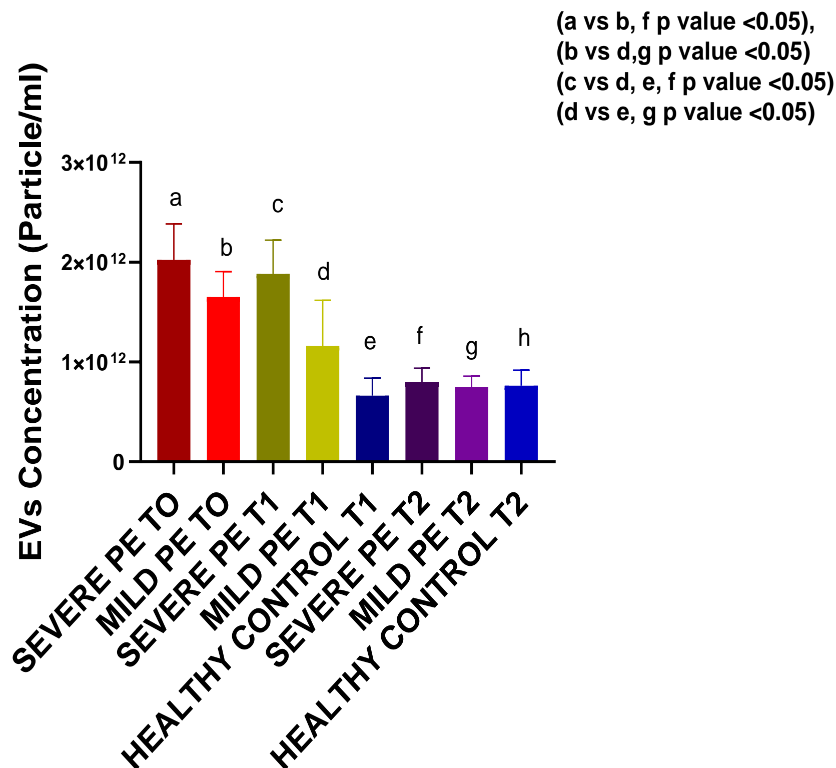


Figure 6: EVs concentration (particle /ml) in severe and mild preeclamptic patients (PE) and healthy controls at different timings. The P values ($p<0.05$) indicates the significance difference between the groups. Reported data are mean ± SD of repeated experiments.

As shown in **Figure 7**, EVs size of preeclamptic patients is higher than that of healthy controls at T1 and T2. In addition, EVs Size is not significantly different in **severe preeclamptic patients among different timings (Figure 7).**

Instead, in mild **preeclamptic patients, we found that EVs size is higher at T2** as compared to that observed at **T0.**

Moreover, the comparison between severe and mild preeclamptic patients shows that **EVs size is higher in severe patients at T0 as compared to mild T0, only.**

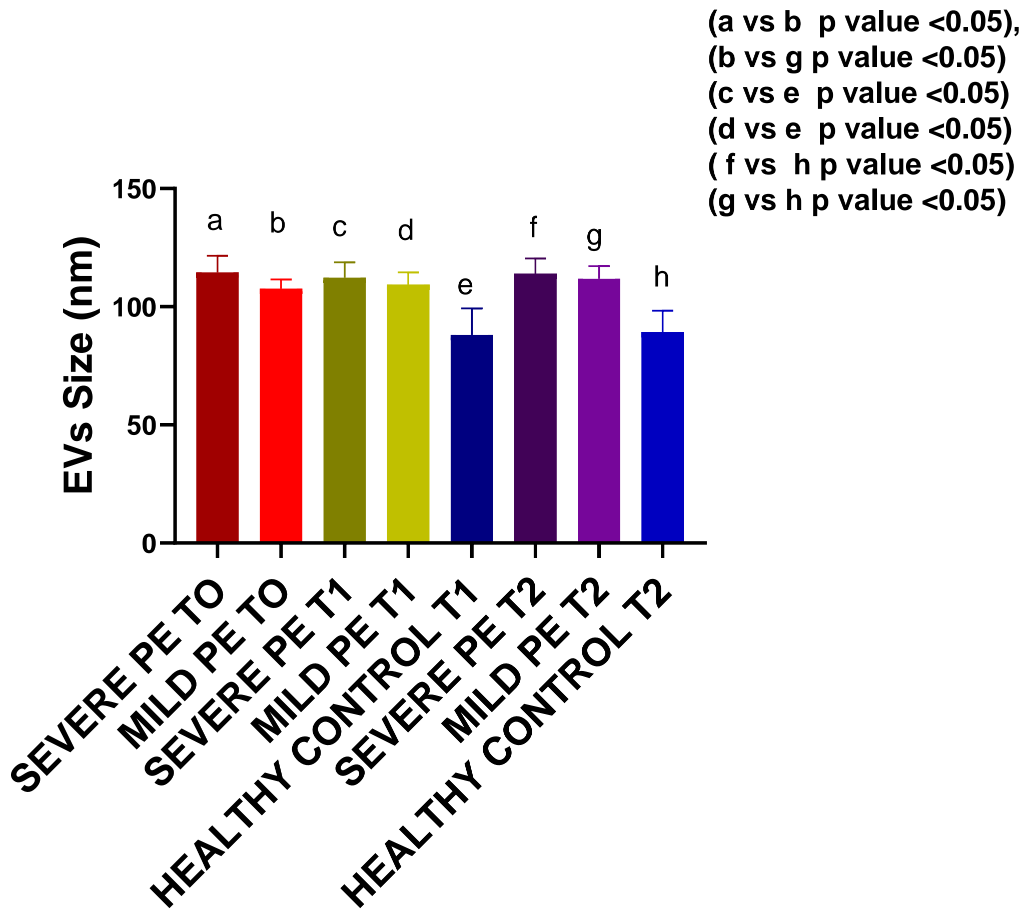


Figure 7: EVs size (Particle /ml) in severe and mild preeclamptic patients (PE) and healthy controls at different timings. The P values ($p<0.05$) indicate the significance difference between the groups. Reported data are mean ± SD of repeated experiments.

Thereafter, we measured exosomal markers on the EVs surface both in patients and healthy controls by using **MACPLEX exosome kit**.

As shown in **Figure 8**, EVs of both patients and healthy control show the expression of **exosomal markers.**

In addition, the expression of all exosomal markers is higher in preeclamptic patients than in healthy controls, at T1. Only CD63 expression is higher in preeclamptic patients than in healthy control at T2. Instead, at T2 CD81 expression in healthy control is higher than in preeclamptic patients.

Also, **CD63** and **CD81** expression is higher in **severe preeclamptic** patients **at T0** as compared to **T1, T2** (**Figure 8A and B).** In addition, **CD 9** expression is higher in **severe preeclamptic** patients **at T0** as compared to **T2, only (Figure 8 C).**

The comparison between severe and mild preeclamptic patients shows that **CD63** expression is higher in **severe preeclamptic patients at all timings,** whereas **CD81** expression is higher in **severe preeclamptic patients as compared to mild preeclamptic patients at T0, only.**

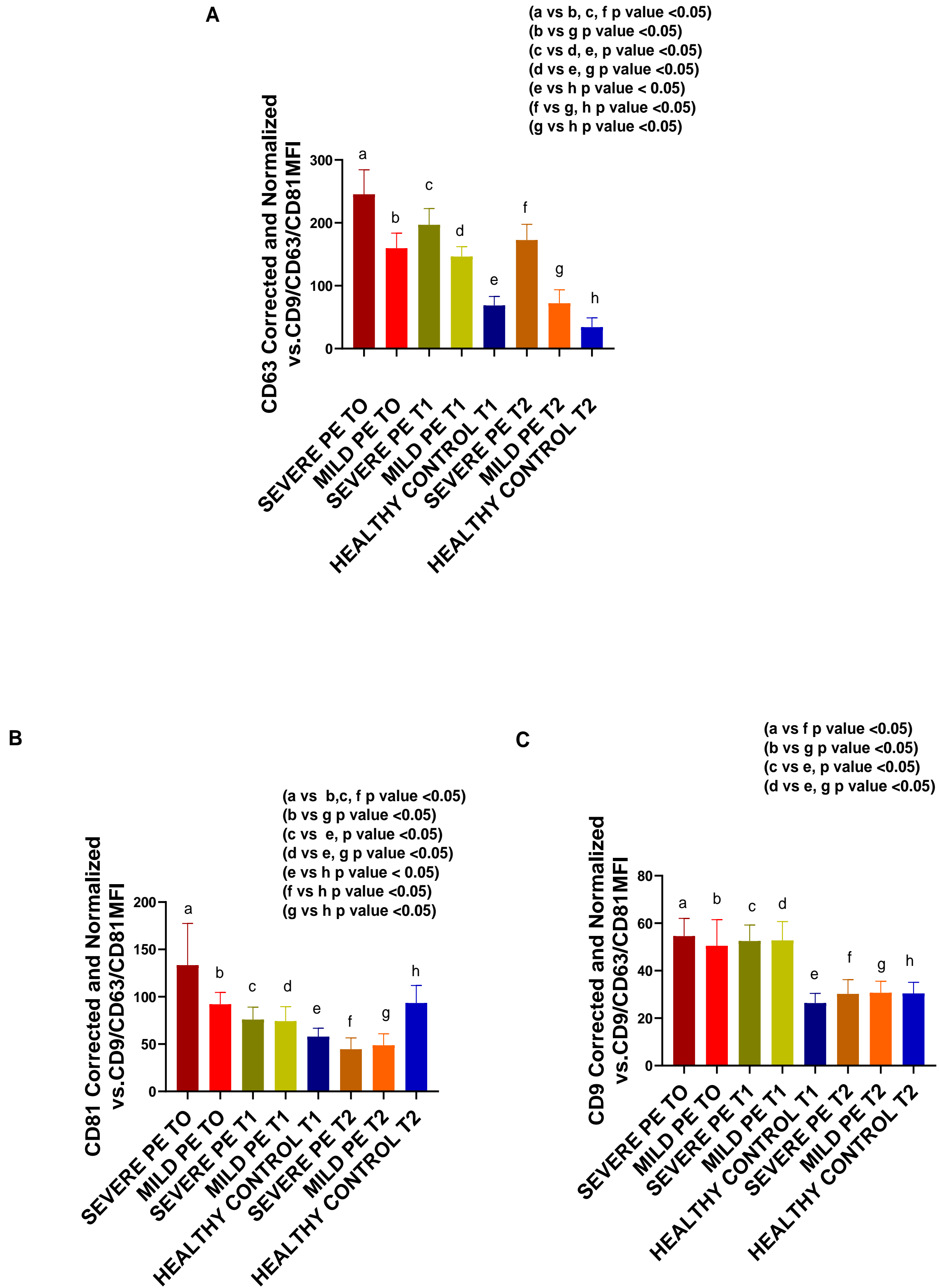


Figure 8. Exosomal markers expression in EVs (CD63, A; CD81, B; CD 9, C) in severe and mild preeclamptic patients (PE) and healthy controls at different timings. The P values ($p<0.05$) indicate the significance difference between the groups. Reported data are mean ± SD of repeated experiments.

In Figure 9, hematopoietic markers expression is shown.

Both **HLA-1** and **CD19** expression is higher in preeclamptic patients than healthy controls at T1 and T2.

Also, **HLA1** and **CD19** expression is higher in **severe preeclamptic patients at T0** as compared to **T1, T2** (**Figure 9 A and B).**

Both marker expressions are also higher in **mild preeclamptic patients at T0** as compared to **T1 T2.**

The comparison between severe and mild preeclamptic patients shows that **HLA 1** expression is higher in **severe preeclamptic patients** as compared to **mild at T0 T1,** whereas **CD19** expression is higher in **severe preeclamptic patients as compared to mild preeclamptic patients at all timings.**

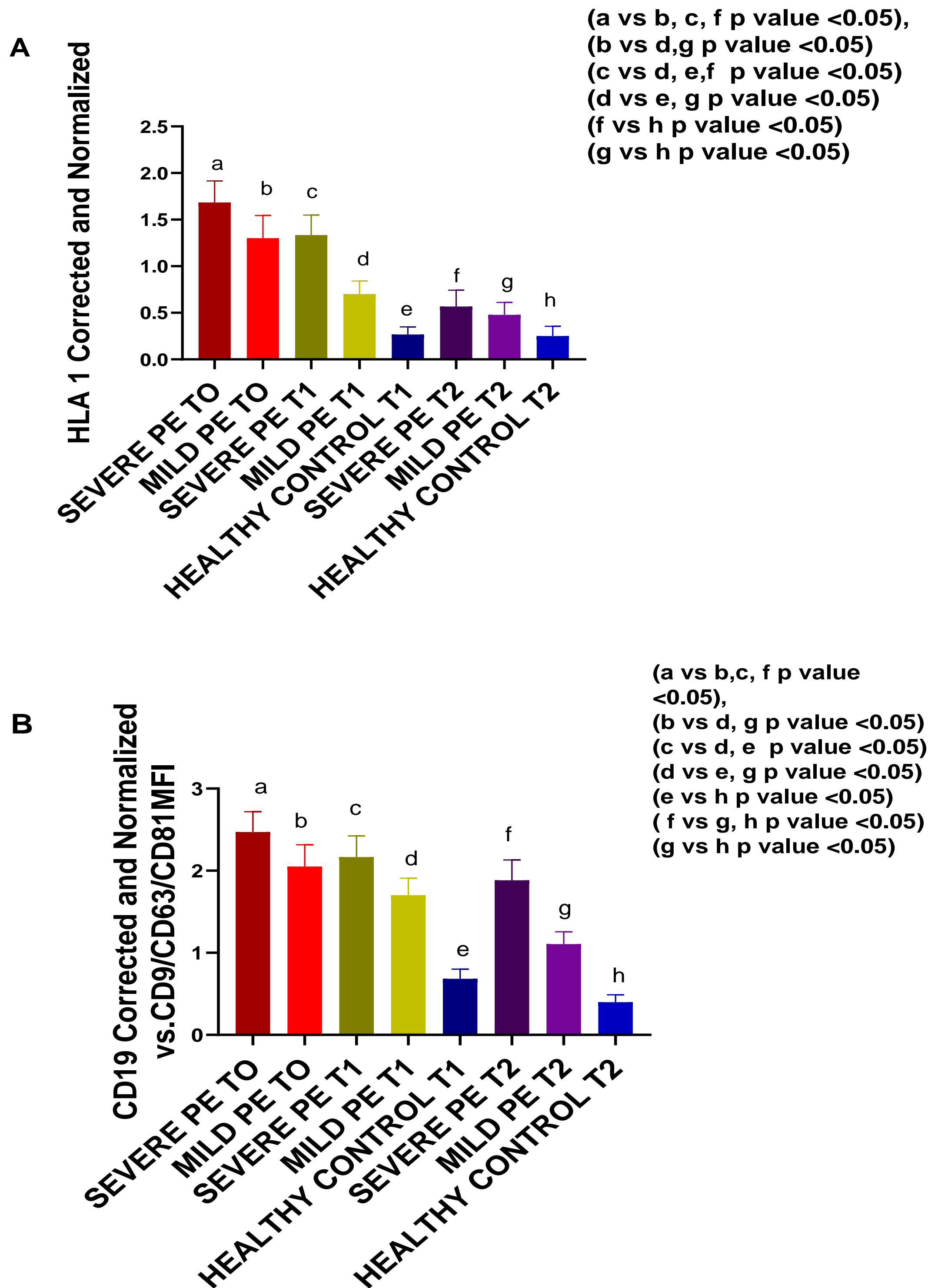


Figure 9: Hematopoietic markers expression in EVs (HLA 1; A and CD19; B) in severe and mild preeclamptic patients (PE) and healthy controls at different timings. The P values ($p<0.05$) indicate the significance difference between the groups. Reported data are mean ± SD of repeated experiments

In Figure 10, the expression of **Lymphocytes markers CD8, CD4** and **CD3** in EVs is shown.

All markers expression is higher in preeclamptic patients than healthy controls at T1 and T2.

As shown in **Figure 10 (A and C)**, **CD8** and **CD3** expression is higher in **severe preeclamptic patients at T0** as compared to **T2**. In addition, **CD4** expression is higher in **severe preeclamptic patients at T0** as compared to **T1, T2 (Figure 10 B).**

In the same way, **CD8** and **CD3** expression is higher in **mild preeclamptic patients at T0** as compared to **T2**. Whereas **CD4** expression is higher in **mild preeclamptic patients at T0** as compared to **T1, T2.**

Finally, the comparison between severe and mild preeclamptic patients shows that **all marker expression** is higher in **severe preeclamptic patients** as compared to **mild patients at all timings.**

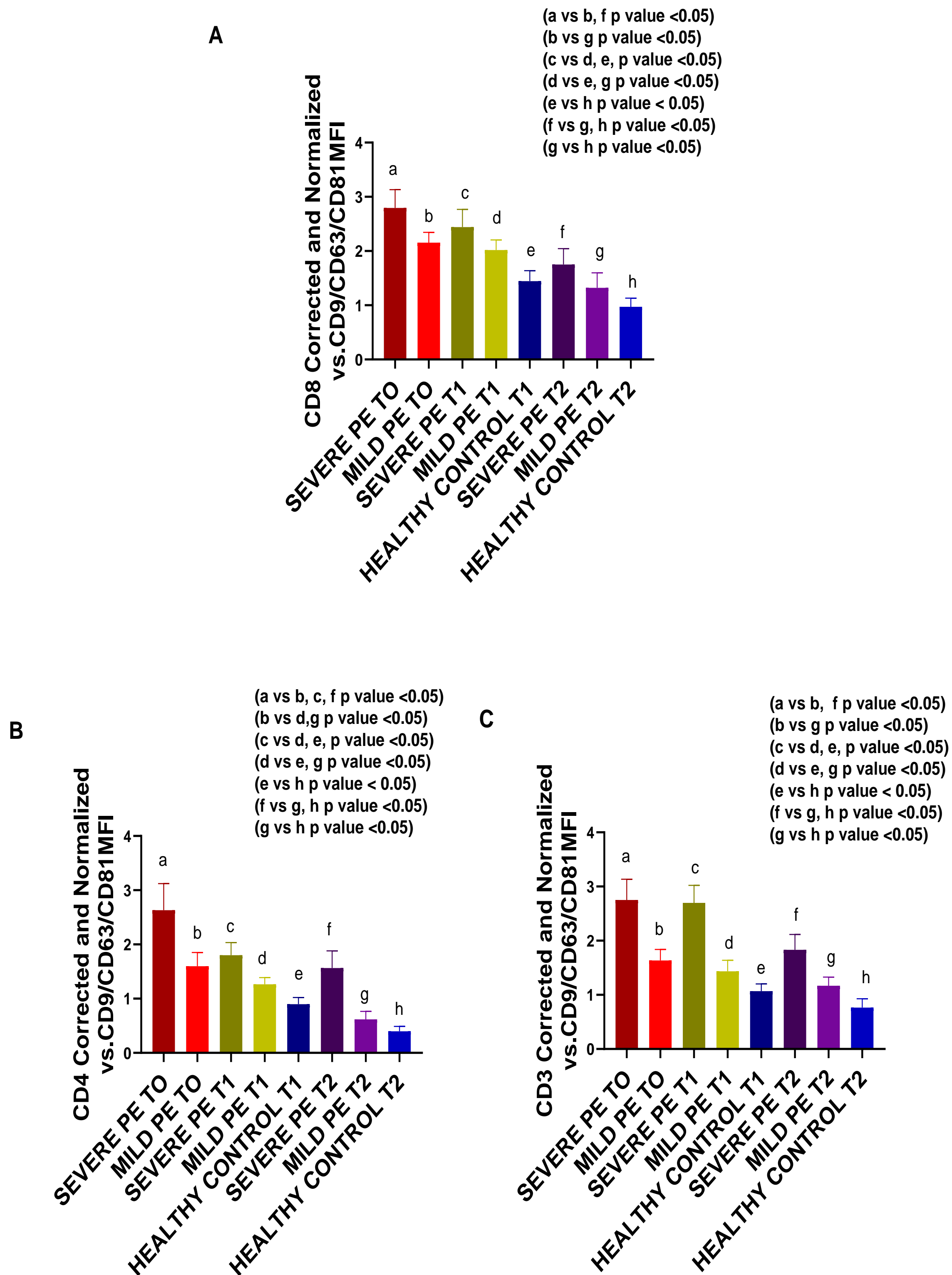


Figure 10: Lymphocytes markers expression in EVs (CD8; A, CD4; B, CD 3; C) in severe and mild preeclamptic patients (PE) and healthy controls at different timings. The P values ($p<0.05$) indicate the significance difference between the groups. Reported data are mean ± SD of repeated experiments

In Figure 11, the expression of **Lymphocytes markers CD20, CD14** and **CD178** in EVs is shown.

All markers expression is higher in preeclamptic patients than healthy controls at T1 and T2.

As shown in **Figure 11**, **CD14, CD178 and CD20** marker expression is higher in severe preeclamptic patients **at T0** as compared to **T1** and **T2.**

Also, **CD20, CD14** and **CD178** expression is higher in **mild preeclamptic patients at T0** as compared to **T1**, **T2 (Figure11).**

The comparison between severe and mild preeclamptic patients shows that **all marker** expression is higher in **severe preeclamptic patients** as compared to **mild patients at all timings.**

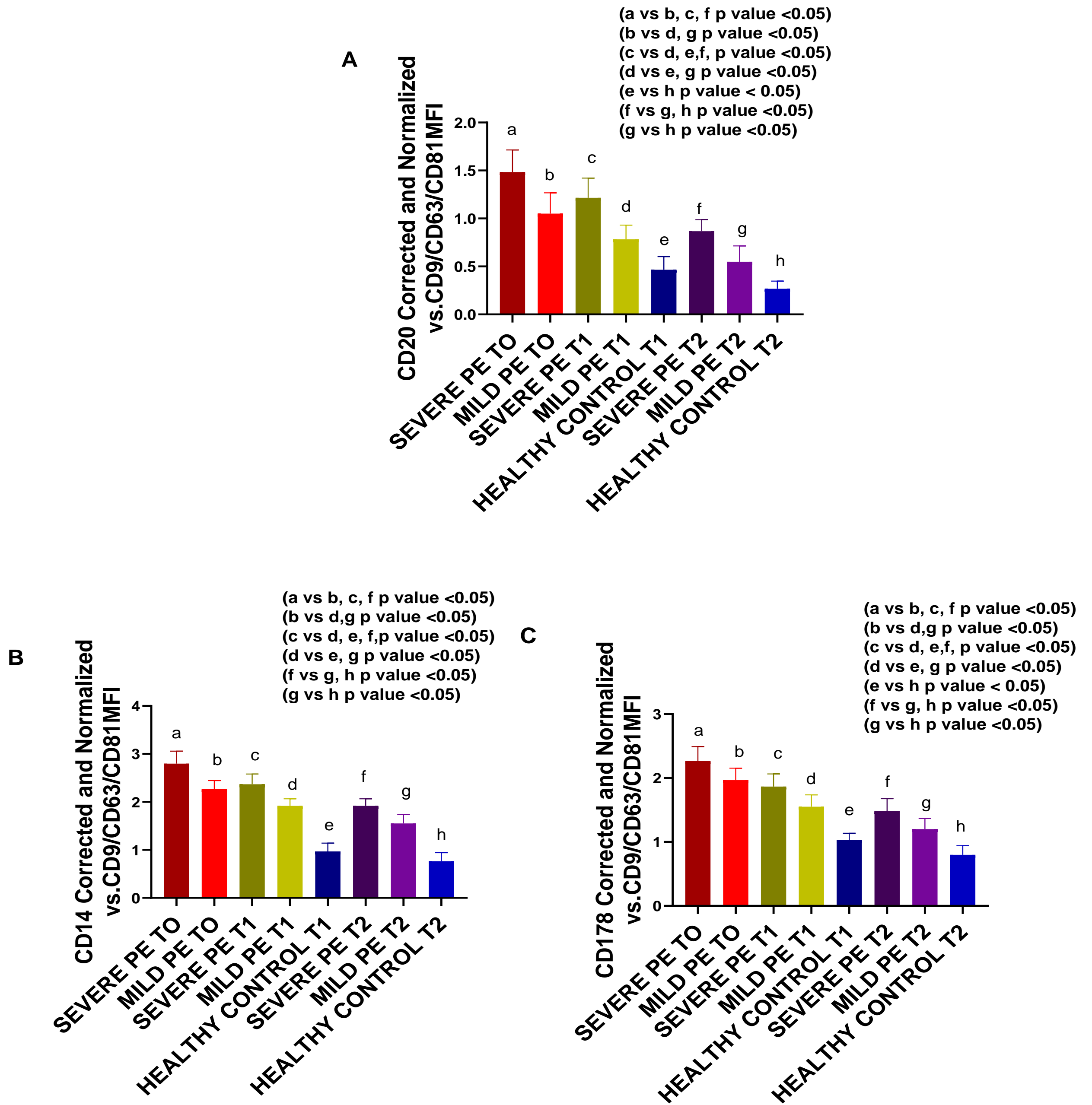


Figure 11: Lymphocytes markers expression in EVs (CD20; A, CD14; B, CD 178; C) in severe and mild preeclamptic patients (PE) and healthy controls at different timings. The P values ($p<0.05$) indicate the significance difference between the groups. Reported data are mean ± SD of repeated experiments.

In Figure 12 the expression of **PLAP and Tissue Factor** in EVs is shown.

Both markers expression is higher in preeclamptic patients than healthy controls at T1 and T2.

As shown in **Figure 12 (A and B)**, **PLAP** and **Tissue factor** marker expression is higher in **severe preeclamptic patients at T0** as compared to **T1, T2.**

In a similar way, **PLAP** and **Tissue factor** marker expression is higher in **mild preeclamptic patients at T0** as compared to **T1, T2.**

The comparison between severe and mild preeclamptic patients shows that **all marker** expression is higher in **severe preeclamptic patients** as compared to **mild preeclamptic patients in all timings.**

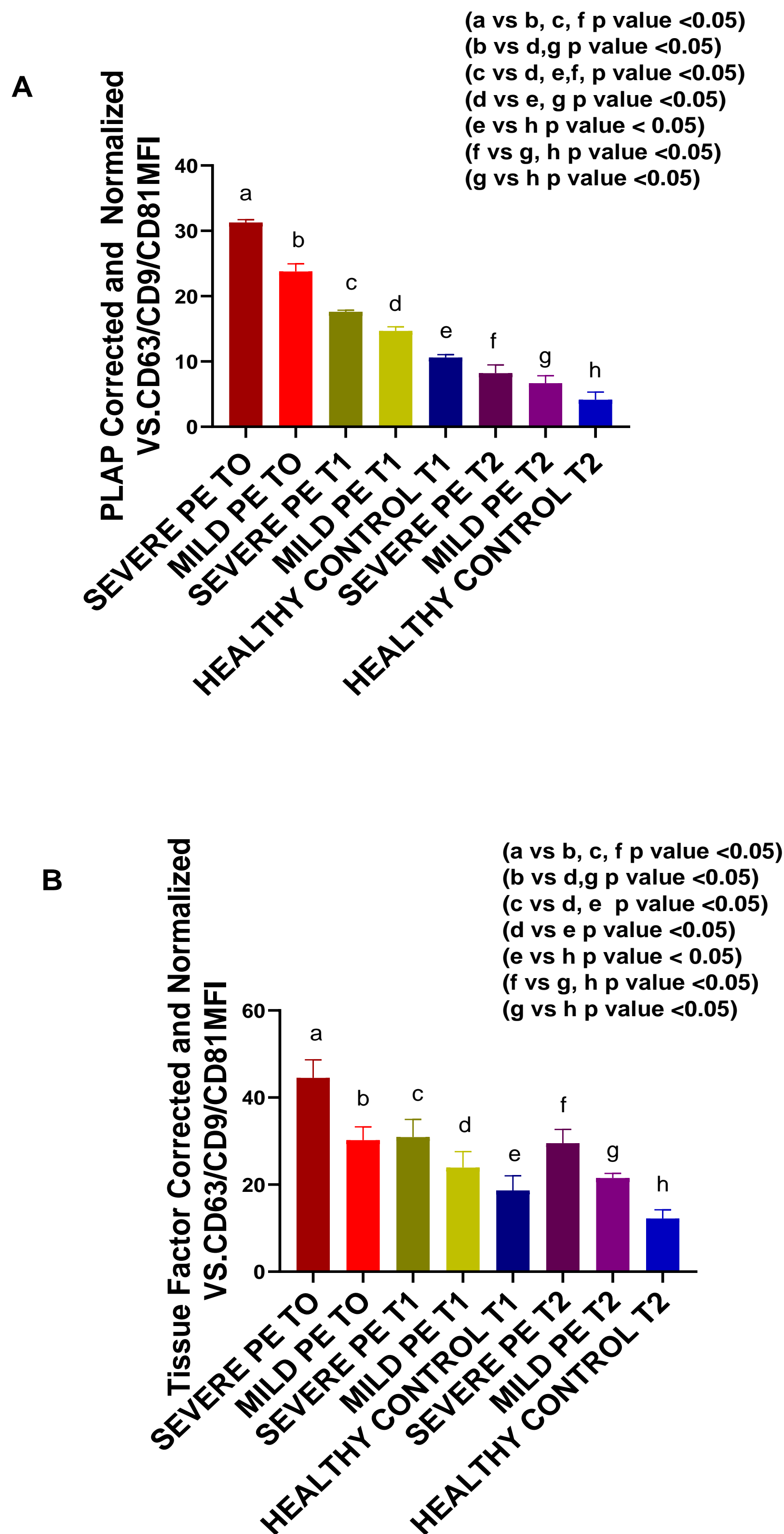


Figure 12: PLAP and Tissue factor markers expression in EVs (PLAP; A, and Tissue Factor; B) in severe and mild preeclamptic patients (PE) and healthy controls at different timings. The P values ($p<0.05$) indicates the significance difference between the groups. Reported data are mean ± SD of repeated experiments.

In Figure 13 the expression of **CD90, CD105** and **CD42b** in EVs, is shown.

All markers expression is higher in preeclamptic patients than healthy controls at T1. As regarding the comparison with T2, all marker expression is higher in preeclamptic patients than healthy control, except CD90 in mild patients.

As shown in **Figure 13**, **CD90, CD105** and **CD42b** marker expression is higher in **severe preeclamptic patients at T0** as compared to **T1, T2.**

Similarly, **CD90, CD105** and **CD 42b** marker Expression is higher in **mild preeclamptic patients at T0** as compared to **T1, T2.**

The comparison between severe and mild preeclamptic patients shows that **all marker** expression is higher in **severe preeclamptic patients** as compared to **mild preeclamptic patients in all timings.**

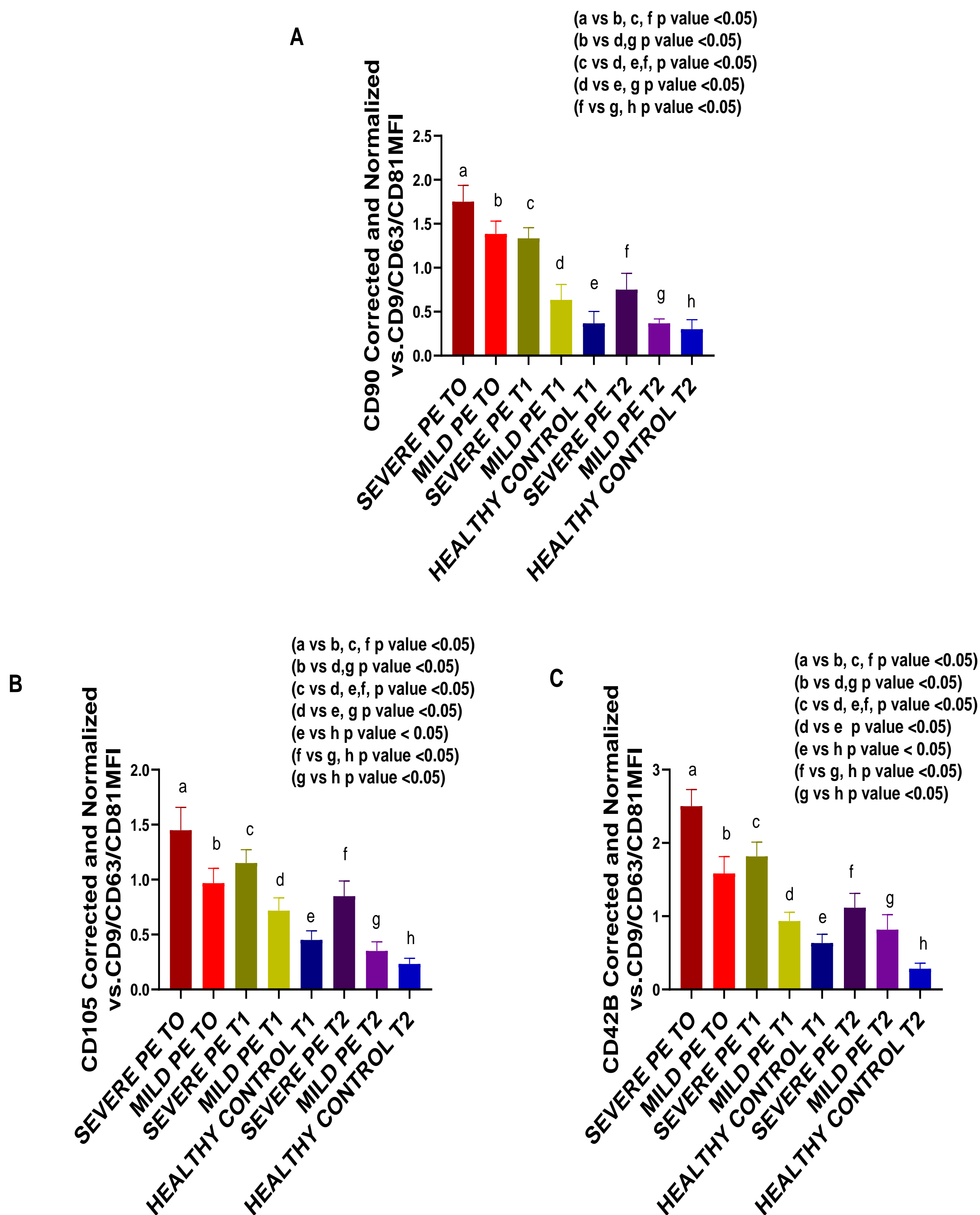


Figure 13: CD90 (Mesenchymal marker; A), CD105 (Endothelial marker; B) and CD42b (Platelets marker; C) markers expression in EVs in severe and mild preeclamptic patients (PE) and healthy controls at different timings. The P values ($p<0.05$) indicates the significance difference between the groups. Reported data are mean ± SD of repeated experiments.

In Vitro Experiments

Thereafter, we analysed the effects of the EVs of preeclamptic patients and healthy controls on **GEC** and **podocytes** by measuring **Cell viability, ROS release** and **Albumin permeability**. In addition, we examined **NO release and Endothelin 1 release in GEC**, as well. In addition, **Nephrin release, and VEGF release** were evaluated in **podocytes**.

As depicted in **Figure 14 A,** there was a reduction of **cell viability in GEC** treated with **EVs** of **preeclamptic patients at all timings** in comparison with **untreated cells**.

Among **severe** and **mild preeclamptic** patients, we found a trend towards an improvement of cell viability from **T0 to T2**.

In addition, cell viability of GEC treated with **severe preeclamptic** patients EVs was always lower than that observed in **mild preeclamptic** patients.

As depicted in **Figure 14 B**, there was a reduction of **Endothelin 1 release in GEC** treated with **EVs** of **preeclamptic patients at all timings** in comparison with **untreated cells**.

No significant differences were found between severe and mild preeclamptic patients at all timing as regarding Endothelin 1 release, except at T2 when in severe patients the Endothelin 1 release was higher.

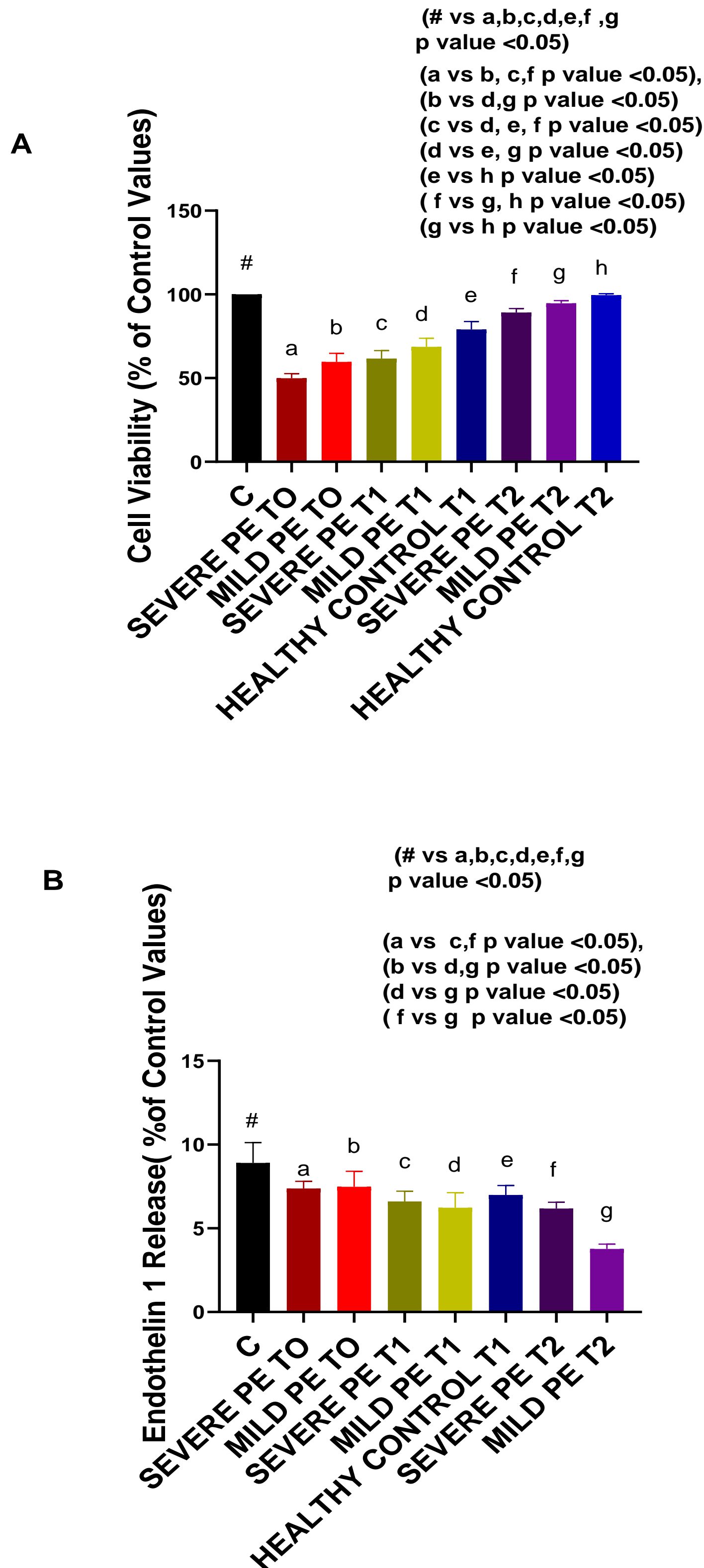


Figure 14: Effects of EVs on GEC. Cell viability (A) and Endothelin 1 release (B) in severe and mild preeclamptic patients (PE) and healthy controls at different timings. C; non-treated cells. The p values ($p<0.05$) indicate the significance difference between the groups. Reported data are mean ± SD of repeated experiments.

As depicted in **Figure 15,** in GEC treated with EVs of preeclamptic patients, **ROS, NO release** and **albumin permeability** were higher than those found in **untreated cells** at all timings.

Among severe and mild preeclamptic patients, we found a trend toward an increase of **ROS release up to T1 in severe preeclamptic patients**, then ROS release was reduced at **T2**.

As regarding **NO release (Figure 15 B),** we found increased NO release from **T0 to T2** in GEC treated with EVs from both severe and mild preeclamptic patients. There were no significant differences between the various timings and among patients.

As regarding the **albumin permeability**, we found that it was increased in all preeclamptic patients with a trend toward the reduction from **T0 to T2**. In addition, we observed significant differences between mild and severe preeclamptic patients at all timings **(Figure 15 C).**

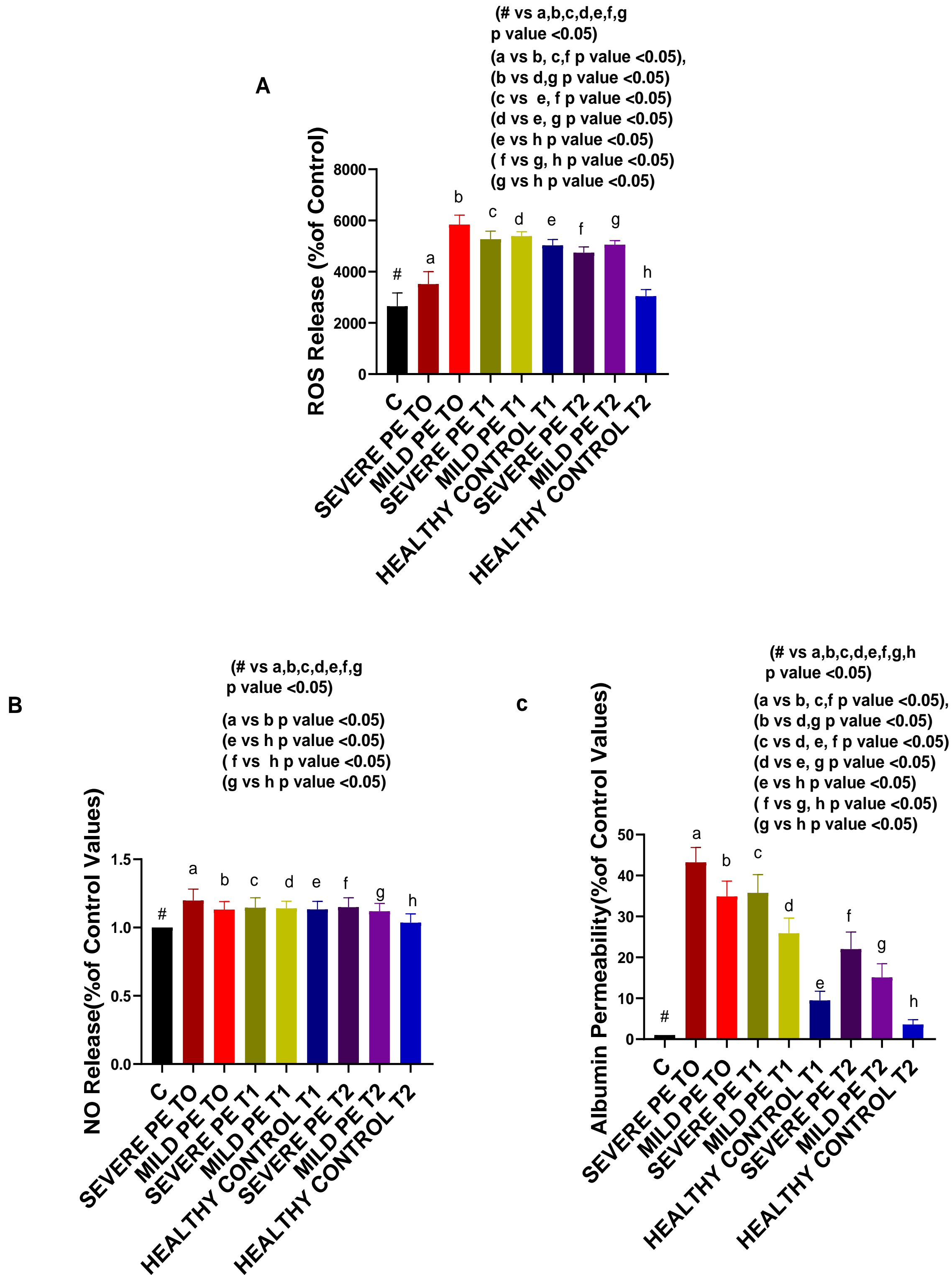


Figure 15: Effects of EVs on GEC. ROS release (A), NO release (B) and Albumin permeability (C) in severe and mild preeclamptic patients (PE) and healthy controls at different timings. C:non-treated cells. The p values ($p<0.05$) indicate the significance difference between the groups. Reported data are mean ± SD of repeated experiments.

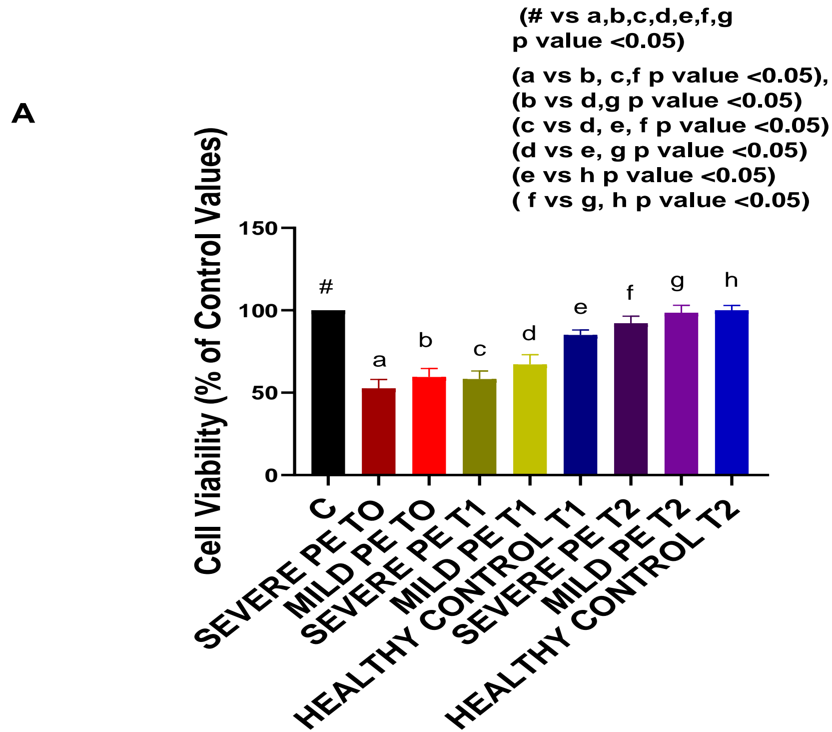
As depicted in **Figure 16 A,** there was a reduction of **cell viability in podocytes** treated with EVs of **preeclamptic patients at all timings** in comparison with **untreated cells**.

Among **severe** and **mild preeclamptic patients**, we found a trend towards an improvement of cell viability from **T0 to T2**.

In addition, **cell viability of podocytes** treated with **severe preeclamptic patients** EVs was always lower than that observed in **mild preeclamptic** patients.

As depicted in **Figure 16 B**, we found an increase of **VEGF release** in **podocytes** treated with EVs of **preeclamptic patients at all timings** in comparison with **untreated cells**.

In addition, **VEGF release of podocytes** treated with **severe preeclamptic patients** EVs was lower than that observed in **mild preeclamptic patients at T1 and T2**.



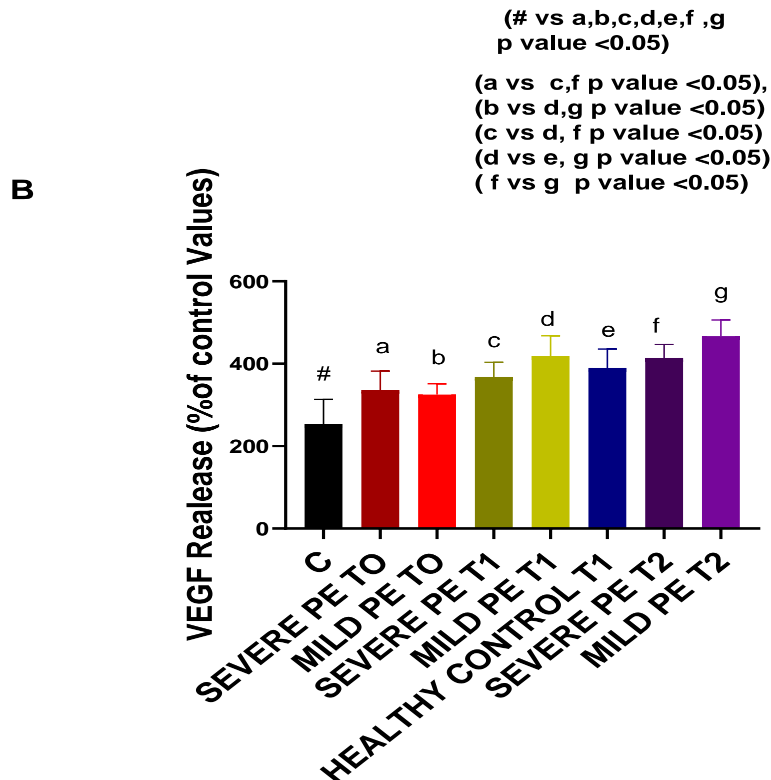


Figure 16: Effects of EVs on podocytes. Cell viability (A) and VEGF release (B) in severe and mild preeclamptic patients (PE) and healthy controls at different timings. C:non-treated cells. The p values ($p<0.05$) indicate the significance difference between the groups. Reported data are mean ± SD of repeated experiments.

In the same time, **ROS,** and **albumin permeability** were higher in p**odocytes** treated with EVs of **preeclamptic patients at all timings** in comparison with **untreated cells** (**Figure 17 A and C).**

Among **severe** and **mild preeclamptic patients**, we found a trend toward reduction of ROS release up to T1 **(Figure 17 A).**

As regarding **Nephrin release (Figure 17 B)**, it was lower in **podocytes** treated with EVs of **preeclamptic patients at all timings** in comparison with **untreated cells.**

In addition, we found the lowest Nephrin release in severe preeclamptic patients at **T0.** Thereafter, Nephrin release increased in both severe and **mild preeclamptic patients (Figure 17 B).**

As regarding the **albumin permeability**, we found that it was increased in all preeclamptic patients with a trend of a reduction from T0 to T2. In addition, we observed significant differences between mild and severe preeclamptic patients **(Figure 17C).**

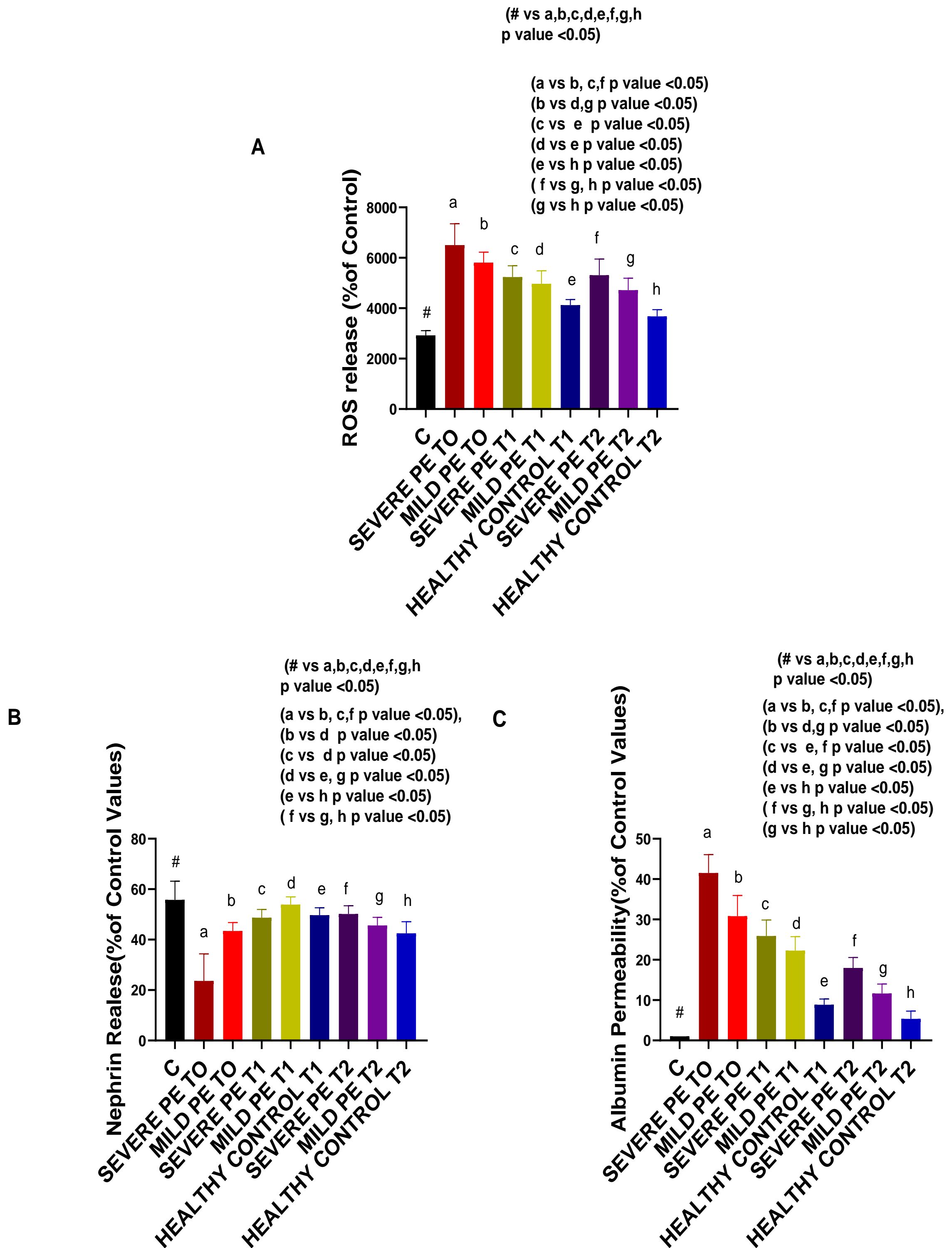


Figure 17: Effects of EVs on podocytes. ROS release (A), Nephrin release (B) and Albumin permeability (C) in severe and mild preeclamptic patients (PE) and healthy controls at different timings. C: non-treated cells. The p values ($p<0.05$) indicate the significance difference between the groups. Reported data are mean ± SD of repeated experiments.

DISCUSSION

DISCUSSION

EVs are cell membrane-surrounded vesicles that are formed during cell activation, apoptosis, and cellular stress. They play a crucial role in intercellular communication by transmitting messages to adjacent and distant cells. EVs are produced under both pathological and physiological conditions and are involved in various physiological and pathological events. The cytoplasm of EVs contains bioactive molecules such as proteins, lipids, signalling molecules, mRNA, microRNA, long non-coding RNAs, and even DNA, which regulate cellular function as paracrine or autocrine vehicles in the microenvironment. EVs mediate inflammation, thrombosis, and immune responses, and their contents can change in various disease conditions, making them potential biomarkers for diseases such as preeclampsia, lupus nephritis, diabetic nephropathy, and others.[54]

EVs are heterogeneous and can carry membranous surface markers of their origin cell, making them a fingerprint of their parent cells in different pathophysiological situations. They are smaller than apoptotic bodies but larger than exosomes and can be found in various biological fluids and tissues. Understanding the roles of specific types of EVs in the pathogenesis of diseases, such as preeclampsia may enable the development of biomarkers to identify individuals at risk for developing these conditions.[55]

EVs play a significant role in the physiopathology of preeclampsia by contributing to the progression of normal pregnancy and potentially initiating the cascade of events that result in preeclampsia. Under both pathological and physiological conditions, different kinds of vesicles are produced, and depending on pre-existing maternal conditions, various vascular components during pregnancy may be capable of initiating the cascade of events that result in preeclampsia. EVs can augment inflammation, coagulation, and endothelial dysfunction in maternal conditions associated with the activation of vascular endothelial cells and immune system modulation.[56] Additionally, placental trophoblast-derived EVs may contribute to the maternal milieu that favours progression to preeclampsia. development of a panel of biomarkers to identify pregnant women at risk for developing preeclampsia. [57]

The study aimed to characterize EVs isolated from plasma of preeclamptic women at different timings during pregnancy (Diagnosis, T0), at delivery (T1) and 1 month after delivery(T2) and to examine their effects on podocytes and GEC. Comparison was made with healthy controls at T1, T2 and non-treated cells.

The study involved the isolation and analysis of EVs using Nano Sight equipped with Nanoparticle Tracking Analysis (NTA) & NTA Analytical Software and the use of MACSPLEX and FACS.

Our findings demonstrate that the concentration of EVs is higher in severe preeclamptic Patients at T0 compared to other timings and in comparison, with mild preeclamptic patients and healthy controls. This suggests a potential correlation between the severity of preeclampsia and the concentration of EVs. The higher concentration of EVs in severe preeclampsia may

reflect the pathophysiological processes associated with the condition, such as endothelial dysfunction, inflammation, and vascular dysfunction

Also, the higher EVs size observed in patients with mild preeclampsia at T0 compared to T2, as well as the higher EVs size in severe preeclampsia at T0 compared to mild preeclampsia at T0, may indicate distinct pathophysiological processes occurring at different stages of the disease. These differences in EVs size could reflect changes in the release and composition of EVs, potentially influencing their effects on podocytes and GEC.

The measurement of exosomal markers on the surface of EVs using the MACPLEX exosome kit, revealed that CD63, CD81, and CD9 were expressed in EVs of patients and controls.

As the first step, Tetraspanins (TSPANs) were evaluated in our samples in order to establish that our EVs were of exosomal origin.

Tetraspanins (TSPANs) are transmembrane proteins including CD63, CD81 and CD 9 found in all cell types and tissues of eukaryotes. They are involved in a large variety of biological processes including cell adhesion and mobility, cell signalling, protein trafficking, cell proliferation, neurotransmission, immune activation, reproduction and others. These proteins, members of the transmembrane 4 superfamily, contribute importantly to cell-cell communication and trafficking of organelles. They are implicated in a large variety of human diseases, including viral and bacterial infections, inflammatory and metabolic diseases (hepatitis, diabetes,) and cancers.[58]

Tetraspanin proteins are used mostly as markers of extracellular vesicles. [59]

The results obtained that, EVs were positive for the typical exosome markers, CD9, CD63, and CD81.

Moreover, all the plasma-EVs samples resulted positive for the surface markers typical of various cell types.

The study compared the expression of lymphocyte/platelet/endothelial markers in EVs isolated from preeclamptic women at different stages of the disease and healthy controls.

The association between the role of EVs and preeclampsia is evident in the differential expression of CD3, CD4, CD8, CD20, CD14 and CD178, indicating potential implications for the immune response and inflammation in preeclampsia. The higher expression of lymphocyte markers in severe preeclampsia suggests a more pronounced immune response and inflammatory state in comparison to mild preeclamptic patients and healthy controls.

These findings contribute to the understanding of the pathophysiology of preeclampsia and the potential role of EVs in modulating immune and inflammatory processes in the disease.

The study compared also the expression of PLAP (Placental alkaline phosphatase), Tissue factor, CD90, CD105, and CD42b in EVs from severe and mild preeclamptic patients at different timings, as well as healthy controls. The findings indicate that these markers have higher expression in both severe and mild preeclampsia at T0 compared to T1, T2, and healthy

controls at T1, T2. Additionally, severe preeclampsia shows more expression of these markers compared to mild preeclamptic patients.

The association between the role of EVs and preeclampsia is evident in the differential expression of these markers, suggesting potential implications for placental function, endothelial dysfunction, and immune modulation in the disease. The higher expression of these markers in severe preeclampsia indicates a more pronounced impact on EV composition and potentially reflects the severity of the disease.

The results obtained about the effects of EVs from preeclamptic patients and healthy controls on GEC and podocytes are particularly significant in the context of the physiopathology of kidney damage.

GEC dysfunction is a central feature of preeclampsia. [60] Our study measured various cellular responses, including cell viability, ROS release, albumin permeability, NO release, Endothelin 1 release in GEC.

The results we obtained shows that EVs of preeclamptic patients were harmful for GEC since they caused the reduction of cell viability and Endothelin 1 release and increased of ROS, NO release and Albumin permeability.

Also in podocytes we found that EVs of preeclamptic patients were harmful. The tests used to examine the effects of EVs on podocytes and in preeclamptic patients included measuring cell viability, ROS release, albumin permeability, Nephrin release and VEGF release.

Overall, the results of the *in vitro* experiments revealed a lower cell viability in both GEC and podocytes when treated with EVs isolated from preeclamptic patients compared to untreated cells. This reduction in cell viability was observed at all timings, indicating a consistent impact of EVs on the viability of those renal cell lines. Additionally, the study found that the cell viability of podocytes treated with EVs from severe preeclamptic patients was consistently lower than that observed in podocytes treated with EVs from mild preeclamptic patients. This suggests a potential correlation between the severity of preeclampsia and the detrimental effects of EVs on cell viability in podocytes.

The reduction in Endothelin-1 release in GEC treated with EVs from preeclamptic patients suggests a potential impact of these vesicles on the endothelial function of the glomerulus. Endothelin-1 is a potent vasoconstrictor and plays a crucial role in regulating vascular tone and blood pressure. Therefore, the lower release of endothelin-1 in GEC treated with EVs from preeclamptic patients may indicate a disruption in the normal vascular function of the glomerulus, potentially contributing to the pathophysiology of preeclampsia.

The study found that the release of ROS was higher in both GEC and podocytes when treated with EVs from preeclamptic patients compared to untreated cells at all timings. Additionally, a trend towards an increase in ROS release was observed up to a certain point, followed by a reduction at a later time. These findings suggest a potential impact of EVs on oxidative stress

in both GEC and podocytes, indicating a potential role of EVs in mediating cellular responses and contributing to the pathophysiology of preeclampsia.

Additionally, we found an increase in albumin permeability in both GEC and podocytes between mild and severe preeclamptic patients at all timings, suggesting a potential correlation between the severity of preeclampsia and the impact of EVs on albumin permeability in renal cells.

The findings indicated also higher NO release in GEC treated with EVs from severe and mild preeclamptic patients compared to untreated cells at all timings, with no significant differences observed between the various timings and among patients. The increased NO release by GEC could also be involved in the endothelial damage due to its conversion into peroxynitrites.

VEGF, or vascular endothelial growth factor, is a key regulator of angiogenesis and vascular permeability, and its dysregulation has been implicated in the pathophysiology of preeclampsia. The observed increase in VEGF release by podocytes suggests a potential impact of EVs on the angiogenic and permeability properties of podocytes, which are essential components of the glomerular filtration barrier. Our findings would also suggest the existence of a crosstalk between podocytes and GEC, which could play a role in the onset of renal damage in preeclampsia.

The observed reduction in Nephrin release suggests a potential disruption in the normal expression of Nephrin and of the function of podocytes when exposed to EVs from preeclamptic patients. This finding may reflect again the pathophysiological changes associated with preeclampsia, and the role of circulating EVs, potentially contributing to the development and progression of the condition.

The differences found in GEC and podocytes treated with EVs of preeclamptic patient's vs healthy control, as regarding cell viability, ROS release, albumin permeability, NO release, endothelin 1, and VEGF release suggest the existence of circulating factors in preeclampsia which could be able to cause damages even after delivery. This could represent a physiopathology basis of the long-term side effects of preeclampsia.

In conclusion, the study's results shed light on the complex interplay between EVs and preeclampsia, offering valuable insights for further research and potential clinical applications. The findings contribute to a deeper understanding of the mechanisms underlying preeclampsia and may have implications for the development of diagnostic and therapeutic strategies targeting EV-mediated cellular responses in this condition.

FUTURE PERSPECTIVES

FUTURE PERSPECTIVES

The future perspective of this study lies in its potential to contribute to the development of diagnostic and therapeutic strategies for preeclampsia. By providing insights into the pathophysiology of the condition and the potential role of EVs in mediating cellular responses, the study opens avenues for further research and clinical applications. The differences in the expression and characteristics of EVs in preeclamptic women compared to healthy controls indicate their potential involvement in immune response, inflammation, endothelial dysfunction, and placental function. Understanding these differences may enable the development of biomarkers to identify individuals at risk for developing preeclampsia and contribute to the development of diagnostic and therapeutic strategies for this condition. Additionally, the study's *in vitro* experiments revealed differences in cellular responses between preeclamptic patients and healthy controls, providing further opportunities for investigating potential therapeutic targets and interventions for preeclampsia. Therefore, the future perspective of this study involves leveraging its findings to advance the understanding of preeclampsia and potentially develop novel approaches for diagnosis and treatment

Further perspectives could be focused on:

Biomarker Development: The study's findings can be used to develop biomarkers for identifying individuals at risk for developing preeclampsia. By understanding the differences in EVs composition and characteristics in different stages of preeclampsia, the study can contribute to the development of diagnostic tools for early detection and monitoring of the condition.

Therapeutic Strategies: The insights gained from the study can be utilized to explore potential therapeutic targets and interventions for preeclampsia. Understanding the effects of EVs on cellular responses, including immune response, inflammation, endothelial dysfunction, and placental function, can guide the development of targeted therapies for managing preeclampsia.

Clinical Applications: The study's *in vitro* experiments on GEC and podocytes provide a foundation for further research on the development of clinical interventions for preeclampsia. The findings can be translated into clinical applications aimed at improving patient outcomes and reducing the impact of preeclampsia on maternal and fatal health.

Longitudinal Studies: Future research can build upon the study by conducting longitudinal studies to track the changes in EVs composition and their effects on cellular function throughout the course of preeclampsia. Longitudinal data can provide a deeper understanding of the dynamic nature of EVs in preeclampsia and their potential as prognostic indicators.

Collaborative Research: Collaboration with other research groups and institutions can further expand the scope of the study's findings. By engaging in collaborative research efforts, the study can contribute to a broader understanding of preeclampsia and facilitate the development of multidisciplinary approaches to address the condition.

These modifications can contribute to advancing the understanding and management of preeclampsia, ultimately benefiting maternal and fatal health.

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