Proposition de sujet de thèse UL-U2A

Project 2025

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Exploration of Pro-inflammatory and Adhesion Molecules in Extracellular Vesicles Isolated from Plasma of Diabetic and Non-Diabetic CKD Patients (Across Stages 3 to 5) : Emphasis on Aortic Calcium Score and Marker Levels.

Cardiovascular disease (CVD) is the number one cause of death worldwide. An estimated 18 million people died from CVD in 2019 , accounting for 33% of global deaths. Vascular calcification (VC) has long been a major area of interest in cardiovascular medicine because of its pathological implications. VC refers to the abnormal accumulation of calcium phosphate crystals within blood vessels and heart valves, a process that contrasts with the physiological calcium phosphate crystals which is typically confined to the skeleton and teeth.

The risks contributing to VC can be categorized into traditional and non-traditional factors. Traditional risk factors include advanced age, hypertension, diabetes, smoking and dyslipidemia (Chen and Moe). Non-traditional risk factors comprise inflammation, oxidative stress, and abnormal mineral metabolism (phosphorus and calcium), bone disorders (MBD) deficiencies in calcification inhibitors (klotho, MGP, PPi and fetuin-A) (Lau et al.), accumulation of uremic toxins in CKD, and among other factors. Detection of VC is achieved using imaging techniques, due to the clinical lack of reliable circulating biomarkers. X-rays and 2-dimensional ultrasound can be used to examine macroscopic calcification of the aorta or peripheral arteries (Covic et al., Vezzoli et al.). Other diagnostic techniques include plain radiographs, tomography and scintigraphy. Computed tomography (CT) scans technology is used to quantify coronary and aorta calcification, including (ultrafast CT scans [electron beam CT (EBCT) and newer multi
slice CT (MSCT)] (Moe and Chen et al.). VC can be divided into valve calcification, medial calcification, and intimal calcification depending on the location within the vessel. Valve VC is the most common disorder that affects heart valves, leading to the thickening and the fibrosis of the aortic valve leaflets. On the other hand, intimal VC is associated with atherosclerosis, affecting plaque stability and increasing the risk of thrombosis. Conversely medial VC is a pathological process linked to aging and metabolic diseases, including Chronic Kideny Disease (CKD) and Diabetes Mellitus. Calcification of the medial layer primarily leads to an increase in arterial stiffness. This could have an extremely detrimental effect, especially when considering large arteries, like the aorta. The loss of elasticity and increased stiffness of the aorta can lead to elevated systolic pressure, and increased cardiac work load which can lead to heart failure, left ventricular hypertrophy, and diastolic dysfunction. The presence and extent of calcification, especially the medial form, have been shown to be strong predictors of all-cause and cardiovascular mortality in hemodialysis (HD) patients.

In CKD patients, VC is developed by different coordinated mechanisms including abnormal mineral metabolism , inflammatory pathways, hormonal imbalance, and uremic toxins. Moreover, In CKD patients, cardiovascular events are the leading cause of death with a high incidence of left ventricular hypertrophy (75%) and congestive heart disease (40%). CKD is classified into 5 stages based on glomular filtration rate (GFR) reduction.

VC used to be considered a passive process. Subsequent studies demonstrated that calcification is an active process regulated by cellular signaling pathways, circulating inhibitors of calcification (Fetuin A (FA) and osteoprotegerin (OPG)),hormones, and genetic factors. VC is mediated by the predominant cell type in the media layer of the artery wall, the vascular smooth muscle cells (VSMCs). These cells can trans-differentiate into osteochondrocyte-like cells, exhibiting increased expression of osteoblast chondrocytes markers including Runt-related transcription factor 2 (RUNX2), Tissue Non Specific Alkaline Phosphatase (TNAP), Osteocalcin, and Osteopontin. As VSMCs undergo this transformation, they lose the expression of SMC-specific proteins, such as αSMA and SM22α, and their contractile properties and gain a proliferative synthetic phenotype, and they gain the ability to produce and secrete bone or cartilage-specific ECM proteins, like Osteopontin (OPN), Osteonectin, collagen I/II and Osteocalcin (OCN). The phenotypic switch of VSMCs was evidenced *in vivo* in animals and humans.
These processes occur under the influence of different stimuli that depend on the underlying disease, including: hyperglycemia (in diabetes), hyperphosphatemia (in CKD), oxidative stress and inflammation. VC in CKD is a culmination of several overlapping pathological processes, which is referred to as the “perfect storm” for VC (Towler et al.).
Different studies, including that of Lomashvili’s group, showed that high phosphate level induces medial VSMC calcification using an *ex vivo* rat aorta models. In support with this, high phosphate diet induces medial calcification and accelerates VSMC osteogenic differentiation in a uremic mouse model of CKD. On the other hand, uremic serum is able to increase the mineralization of VSMCs regardless of the serum phosphate concentration (Chen et al.), suggesting that other factors than Pi are involved. Local upregulation of TNF-α in CKD is accompanied with expression of Msx-2 and BMP-2 which are known promoters of osteoblastic trans-differentiation of VSMCs (Koleganova et al.). BMP-2 itself is thought to play a role in the induction of VC in CKD through Wnt-β-catenin pathway (Mizobuchi et al. ; Rong et al.). In addition, nitrotyrosine (oxidative stress marker) immunostaining is increased in the aortas of uremic apoE knock-out mice (Phan et al.) and in the arteries of CKD patients (Guilgen et al.).
Moreover, diabetes and CKD are closely linked, with diabetes being one of the primary factors for CKD progression and development. VC is also increased in patients with diabetes, and is associated with increased morbidity and mortality rates compared to diabetics without calcification. *In vitro* studies have shown that high concentrations of glucose accelerate calcification of Bovine VSMCs (Chen et al). In addition, human aortic smooth muscle cells, treated by high glucose manifest calcification and osteoblastic trans-5 differentiation via the BMP-2/Runx2 pathway, an effect which can be partially blocked by noggin protein, which inhibits BMP-2 (Liu et al.,). Furthermore, in rat VSMCs, glucose increases alkaline phosphatase activity, mineralization and osteocalcin expression (Sinha and Vyavahare,). Finally, it has been shown that high glucose induces murine VSMC mineralization and trans-differentiation into chondrocyte-like cells (Bessueille et al.,). Therefore, a greater understanding of the mechanisms through which diabetes may induce calcification is required in order to develop effective strategies to interrupt this process. Extracellular vesicles (EVs) play a key role in VC. EVs are released into blood stream by many cells under normal and diseased conditions. They perform a pivotal role in regulating vascular homeostasis and participate in different pathological processes including atherosclerosis, dilatation of aorta wall , aortic aneurysm (Han et al 2020) and

Aortic stenosis (Weber et al., 2019). Other authors demonstrated that patients with Aortic stenosis and high Coronary Calcification score exhibited higher levels of EVs associated with thrombin activity when compared with aortic stenosis patients with low coronary calcification score (Hon et al., 2016) demonstrating the impact of EVs in endothelial dysfunction, inflammation, and valvular calcification. In addition, Furmanik et al have demonstrated that the phenotypic switch of SMCs was correlated with Nox-5 expression which enhanced the oxidative stress that led the production of EVs. EVs act as a source of diagnostic information due to their specific composition containing proteins, nucleic acid, and other biomolecules that reflect the physiological or pathological state of the parent cells. EVs are characterized by remarkable stability, protecting their cargo from degradation by endogenous enzymes thus making EVs potential biomarkers with promising applications in disease diagnosis, and prognosis.
In Our project we aim to explore the proinflammatory and adhesion molecules of EVs isolated from the Plasma of Diabetic and non Diabetic CKD patients across stages 3 to 5. Moreover, we will focus on the Aortic calcium Score (ASC) to investigate the relationship between ASC and the level of the EVs biomarkers in the context of aortic vascular calcification progression in Diabetic and non-diabetic CKD patients. The results obtained will provide us with new insights that address therapeutic targets hence predicting treatment responses.

Aims :

1- Analyse the Aortic Calcium Score (ACS) of diabetic and non-diabetic CKD patients across stages 3 to 5.

2- Characterize the EVs isolated from the plasma of diabetic and non-diabetic CKD patients at different stages based on the specific markers CD63, CD81, CD9, Alix,TSG101, and Annexin V.

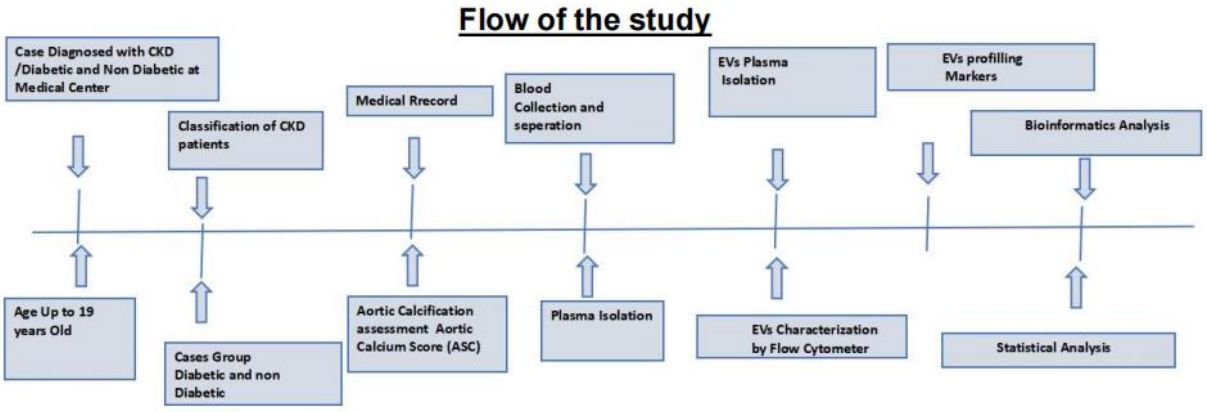
3- Identify the variations of panel of pro-inflammation including GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and TNF-α, and adhesion markers (ICAM-1, VCAM-1 E-Selectin and P-Selectin..) of EVs isolated from the plasma between diabetic and non-diabetic CKD patients at different stages.

4-To determine the involvement of miRNAs in by conducting EVs miRNA profiling to identify the dysregulated miRNAs that serve as biomarkers and the downstream signaling pathways it affects for better treatment strategies.

5- Investigate the potential correlation between ACS and the level of these markers in EVS, in the context of CKd progression and diabetes status.

Material and Methods

Flow of the study



Study Population

This study will focus on CKD patients across the stages from 3 to 5 to examine the differences in VC (Aortic Calcification) and plasma EVs Proinflmmatory and adhesion molecules between these groups: Diabetic CKD (n=30), Non-Diabetic CKD (n=30) and Control Group (Non-CKD) (n=15). The Inclusion criteria are age (adults aged 18 and older), CKD stage, (patients diagnosed with CKD stages 3,4, or 5),treatment (patients receiving hemodialysis treatment) and diabetic status. Both diabetic and non-diabetic patients are included in this study. The Exclusion,criteria are acute illness (patients with acute illnesses or infections), conditions (patients with active cancer or severe heart failure), patients with a history of organ transplantation, acute cardiovascular events within the past 6 months, or other significant comorbidity affecting aortic calcification.

Assessment of Aortic Calcification

Imaging techniques, such as computed tomography (CT) or X-ray, will be utilized to assess the extent and the severity of aortic calcification in the study participants. Scoring systems, such as the Agatston score or the volume-based method, will be applied to quantify the amount of calcification in the aorta. Other imaging modalities like ultrasound or magnetic resonance imaging (MRI) might be used as well to complement the evaluation of aortic calcification.

Human Sample Processing

a- Guidelines:

All human blood samples should be handled according to the Centers for Disease Control (CDC) universal blood and body fluid collection guidelines. Additionally, sample handling and processing should follow the Occupational Safety and Health Administration (OSHA) blood borne pathogens procedures to prevent possible pathogen transmission. All the research should be reviewed and approved by the site-specific Institutional Review Board (IRB) prior to proceeding.

b- Human plasma collection .
1.5 mL of peripheral venous blood is collected by a trained nurse or phlebotomist into prelabeled EDTA-coated evacuated tubes. Immediately after the sample is collected, the tube should be thoroughly mixed and stored at room temperature (< 2 hours). Centrifuge whole blood at 2,500 x g for 15 minutes at room temperature. Collect the topmost layer (plasma) of supernatant into a 15 mL conical tube. Snap freeze plasma fractions and store at -80°C.

Extracellular vesicles EVs Isolation:
Extracellular vesicles (EVs) isolation from the plasma of the study participant using the exoEasy Maxi Kit from Qiagen (cat. no. 76064). The following protocol focuses on the isolation and the quantification of plasma EVs 30–300 nm in diameter (exclude larger Apoptotic bodies, Oncosomes, and smaller Exomeres).
To purify extracellular vesicles (EVs) from plasma using Qiagen kits, begin by collecting blood samples in EDTA tubes and centrifuging them at 2,000 × g for 10 minutes to isolate plasma. Follow this by employing Qiagen EV isolation kits in accordance with the manufacturer's guidelines. This usually involves mixing the plasma with a specific buffer, allowing for incubation, and subsequently carrying out centrifugation or filtration steps to extract EVs. Post-isolation, proceed with enriching the EVs using the columns or beads provided in the kit by adhering to the recommended procedures. Subsequently, conduct washing steps to eliminate contaminants from the EVs through either centrifugation or filtration in accordance with the Qiagen kit instructions. Elute the purified EVs from the columns or beads using the elution buffer supplied in the kit. Finally, store the purified EVs at -80°C for extended preservation.

Extracellular Vesicles (EVs) Plasma Dosing :
Extracellular vesicles (EVs) isolated will be characterized by their size and quantified and analyze using commercially available EVs plasma dosing kits (The Vesicle Flow Cytometry (vFC™) assay,Cellarcus BioSciences). The Vesicle Flow Cytometry (vFC™) assay allows for the detailed characterization of extracellular vesicles (EVs) based on specific markers. Common markers used for EVs characterization through flow cytometry (Beckman Coulter CytoFLEX) include CD63, CD81, CD9, Alix,TSG101, and Annexin V. These markers are play crucial roles in their biogenesis, release, and functions. The vFC™ Assay Kit includes calibrators and standards for instrument performance characterization and calibration.

Biomarkers profiling in Plasma Evs :
Evs proteins as well as pro-Inflammatory Markers (GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and TNF-α,) Adhesion Molecules (ICAM-1, VCAM-1 E-Selectin and PSelectin..),and Vascular Calcification markers, will be assayed using multiplex immunoassays (ThermoFisher Scientific) on a **Luminex Platform**. Differences between the two groups will be made by univariate analysis.

MiRNA profiling:

MiRNA will be extracted from 200 µL of isolated EVs using the miRNeasy Mini Kit (Qiagen, Hilden, Germany, catalog no. 74104) according to the manufacturer's instructions. The RNA quantity and purity will be assessed with the Agilent 2100 Bioanalyzer system (Agilent Technologies, Waldbroon, Germany). Reverse transcription (RT) will be performed using the miRCURY LNA Universal cDNA Synthesis Kit II (Exiqon-Qiagen, Hilden, Germany, catalog no. 203301). Quantitative polymerase chain reaction (qPCR) will be performed using the miRCURY LNA Universal RT microRNA PCR system (Exiqon-Qiagen, catalog no. 339340) with 752 known human miRNAs and 3 interplate calibrators and 1 spike-in miRNA as an internal control.

Statistical Analysis: Statistical methods, such as t-tests, chi-square tests, or nonparametric tests, will be employed to compare the biomarker profiles of plasma EVs between CKD patients that are diabetic and CKD non-diabetic groups. Correlation analysis or regression analysis will be performed to assess the associations between the specific biomarkers and the severity of aortic calcification.