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ABDOMINAL AORTIC ANEURYSM AND CANCER

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ABSTRACT

Abdominal aortic aneurysm (AAA) is a vascular disease characterized by permanent expansion of the infrarenal aorta's diameter.

Multiple clinical trials have indicated that cancer, rather than cardiovascular illnesses, was the leading cause of death in follow-up examinations of AAA patients that underwent repair surgery. Also, inflammation has been proven to play an important role in cancer development. Since AAA includes persistent aortic wall inflammation, a potential link could be made between AAA and cancer.

Based on these two facts, our hypothesis was that aneurysmal tissue would leak inflammatory markers and other molecules like micro-RNAs (miRNAs) into the circulation, which might have a systemic effect and be able to promote cancer. This hypothesis motivated us to search for miRNA candidate biomarker(s) in plasma of AAA patients that developed cancer and that did not. Indeed, miRNAs were identified in the circulation and have since been explored as possible biomarkers for a variety of illnesses.

Our results showed that a specific miRNA stood out among the others. Hsa-miR-122-5p was found significantly upregulated in cancer patients compared to non-cancer patients. This miRNA warrants further exploration with a more extensive experimental design in order to validate its role in AAA and cancer and to establish it as a possible therapeutic target.

RÉSUMÉ

L'anévrisme de l'aorte abdominale (AAA) est une maladie vasculaire caractérisée par une expansion permanente du diamètre de l'aorte infrarénale.

De multiples essais cliniques ont indiqué que le cancer, plutôt que les maladies cardiovasculaires, était la principale cause de décès lors des examens de suivi des patients atteints d'AAA ayant subi un traitement chirurgical. Il a également été prouvé que l'inflammation joue un rôle important dans le développement du cancer. Comme l'AAA comprend une inflammation persistante de la paroi aortique, un lien potentiel pourrait être établi entre l'AAA et le cancer.

Sur la base de ces deux faits, notre hypothèse était que le tissu anévrismal laisserait échapper des marqueurs inflammatoires et d'autres molécules comme les micro-ARN (miRNA) dans la circulation, ce qui pourrait avoir un effet systémique et être capable de promouvoir le cancer. Cette hypothèse nous a motivés à rechercher un ou plusieurs biomarqueurs candidats miRNA dans le plasma de patients atteints d'AAA qui ont développé un cancer et ceux qui n'en ont pas développé. En effet, les miRNA ont été identifiés dans la circulation et ont depuis été explorés comme biomarqueurs possibles pour une variété de maladies.

Nos résultats ont montré qu'un miARN spécifique se distinguait des autres. Le Hsa-miR-122-5p a été trouvé significativement régulé à la hausse chez les patients cancéreux par rapport aux patients non cancéreux. Ce miARN mérite d'être exploré plus en profondeur avec un plan expérimental plus étendu afin de valider son rôle dans l'AAA et le cancer et de l’établir comme une possible cible thérapeutique.

ABBREVIATIONS

AAA = Abdominal aortic aneurysm

BM-MSC = Bone marrow-derived mesenchymal stem cells

CPM = Counts per million

CVD = Cardiovascular disease

DREAM = Dutch Randomized Endovascular Aneurysm Repair

ECM = Extracellular matrix

EDTA = Ethylenediamine tetraacetic acid

EVAR = Endovascular aneurysm repair

HCV = Hepatitis C virus

ILT = Intraluminal thrombus

LNA = Locked Nucleic Acids

MAPK = Mitogen-activated protein kinase

miRNA = Micro-RNA

MMP = Matrix metalloprotease

NF-κB = Nuclear factor-kappa B

NGS = Next-generation sequencing

NSCLC = Non-small cell lung cancer

PCR = Polymerase chain reaction

PTEN = Phosphatase and tensin homolog

QC = Quality control

RISC = RNA-Induced Silencing Complex

RT = Room temperature

RT-qPCR = Quantitative reverse transcription PCR

TAA = Thoracic aortic aneurysm

TGFß = Transforming growth factor beta

TMM = Trimmed mean of M-values

UMI = Unique Molecular Identifier

UTR = Untranslated region

VSMC = Vascular smooth muscle cell

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1 Introduction

1.1 Generalities

An aneurysm is an irreversible and permanent dilatation of an artery affecting all of its three layers ; the intima, the media and the adventitia from the inside to the outside (Sakalihasan et al., 2018). In humans, the abdominal aorta is the most common site for development of aneurysms (Kontopodis et al., 2018). It is considered an abdominal aortic aneurysm (AAA) when the infrarenal artery is affected. Its normal diameter is generally ranging between 18 and 22 mm in men and between 16 and 20 mm in women, while a 1.5 fold increase corresponds to the pathological diameter (Kontopodis et al., 2018). In clinical practice, a threshold of 30 mm of diameter has been established for the definition of an AAA (Sakalihasan et al., 2018).

1.2 Cardiovascular structure and physiology

1.2.1 The vascular system

To sustain metabolic homeostasis, the body requires oxygen, nutrition, and waste removal. The vascular system, which is a network of blood vessels, is responsible for delivering oxygen and nutrition to all organs and tissues while also eliminating waste materials and it makes, together with the heart, which functions as a pump, the cardiovascular system. The vascular system contains 5 classes of vessels. The arteries, with the arterioles, carrying oxygenated blood from the heart, transport oxygen, nutrition, hormones, and other chemicals throughout the body. The veins, with the venules, that leave the organs and tissues bringing metabolic waste back to the heart. And finally, the tiniest blood arteries, capillaries, connect arterioles and venules through networks throughout organs and tissues (Selina, 2018)

1.2.2 Arteries

As mentioned above, arteries deliver oxygen-rich blood to the tissues in a high-pressure system. Elastic arteries, muscular arteries, and arterioles, which are the smallest arteries, are the three types of arteries, and except for the smallest, have 3 layers **(Figure 1)**. Tunica intima which is the inner layer, also called endothelium, directly in contact with the blood flow. Tunica media is the thickest layer of the arteries. It is composed by smooth muscle, elastic fibers, collagen fibers, and fibroblasts. And the outside layer is tunica adventitia. It is mostly made up of connective tissue fibers that protect blood vessels and link them to neighboring tissues. Additional tiny vessels, vasa vasorum, give blood and nutrients to the tunica adventitia and tunica media in bigger blood vessels (Ovalle & Nahirney, 2013; Selina, 2018).

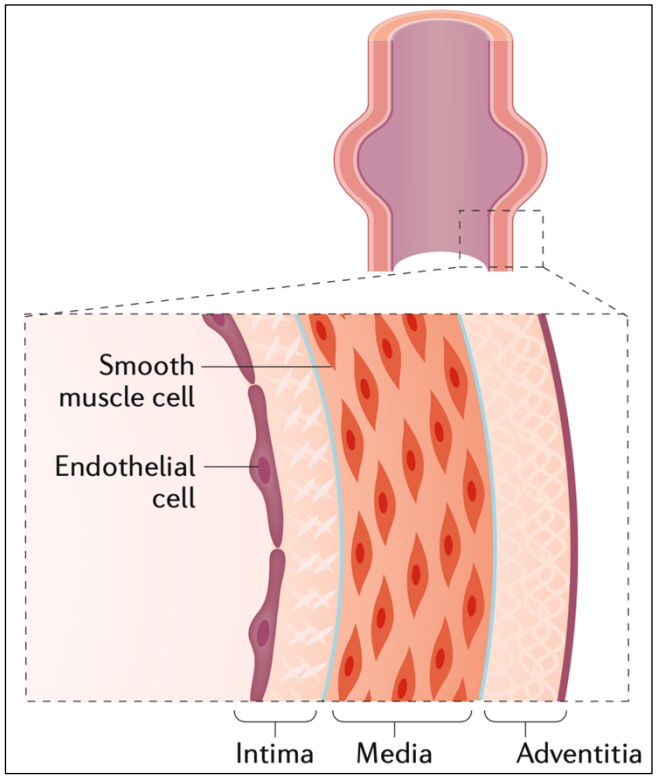

Figure 1: Aortic wall composition. *From Sakalihasan N. et al., 2018*

1.2.2.1 Elastic arteries

Elastic arteries are close to the heart and have the biggest diameter (1-2,5cm) and include a lot of elastin and smooth muscle. They have a big lumen and low blood flow resistance, and they may expand and recoil to meet variations in blood volume. The aorta and pulmonary arteries, as well as the common carotid, subclavian, and common iliac arteries, are all included. They carry blood from the heart to muscle arteries.

The tunica media is the most apparent of three layers in the elastic vessel wall. It features a dense network of elastic fibers structured as several concentric laminae interspersed with smooth muscle cells, arranged in a circular pattern, and collagen. Collagen gives artery walls

tensile strength and help maintain the structural integrity of the vascular wall, while elastic fibers provide them distensibility, allowing passive recoil under pressure**.**

The tunica intima accounts for up to 20% of the wall thickness and its luminal surface is lined inside by endothelial cells lying on a basal lamina. A boundary of an internal elastic lamina runs beneath the intima, which is typically difficult to distinguish since it merges gradually with medial elastic laminae.

The tunica adventitia is made up of loose, irregular connective tissue with a majority of longitudinal collagen fibers and dispersed fibroblasts. Small nutritive vasa vasorum and lymphatic capillaries are found in the adventitia of most elastic arteries. This microvasculature can be found all the way to the media's border. The abdominal aorta is an exception; it lacks vasa vasorum, which might explain its tendency for dilatation and aneurysm development (Ovalle & Nahirney, 2013; Sakalihasan et al., 2005; Selina, 2018; Stevens & Lowe, 1997).

1.2.2.2 Muscular arteries

Large elastic arteries gradually become muscular arteries that control local blood flow and transport blood to specific organs. They have a diameter of 0,3mm to 1cm and have more smooth muscle but less elastin than elastic arteries. They act by contracting and relaxing smooth muscle in their walls and are known as distributing arteries.

The tunica media, the thickest layer of the arteries, is dominated by smooth muscle, giving them their very contractile nature. Variable quantities of elastic fibers, collagen fibers, and fibroblasts are found between smooth muscle layers (Ovalle & Nahirney, 2013; Selina, 2018; Stevens & Lowe, 1997).

1.2.3 Aorta

The thoracic aorta, located in the chest, and the abdominal aorta, located below the diaphragm (the muscle that links the chest to the belly) are the two main parts of the aorta. The aortic root, ascending aorta, aortic arch, and descending aorta are the four major segments of the thoracic aorta. The aortic root is the section of the aorta that connects to the heart and contains the aortic valve as well as the openings for the coronary arteries, which provide direct blood flow to the heart. The ascending aorta begins just above the aortic root and ascends the chest to the head. The aortic arch bends over the heart, supplying blood flow to the brain and limbs by branches. After going through the lungs and diaphragm, the descending aorta transforms into the abdominal aorta with the coeliac trunk, mesenteric and renal arteries. The bifurcation of the aorta into common iliac arteries marks the end of the aorta **(Figure 2)** (Salameh et al., 2018; Zipes et al., 2018)

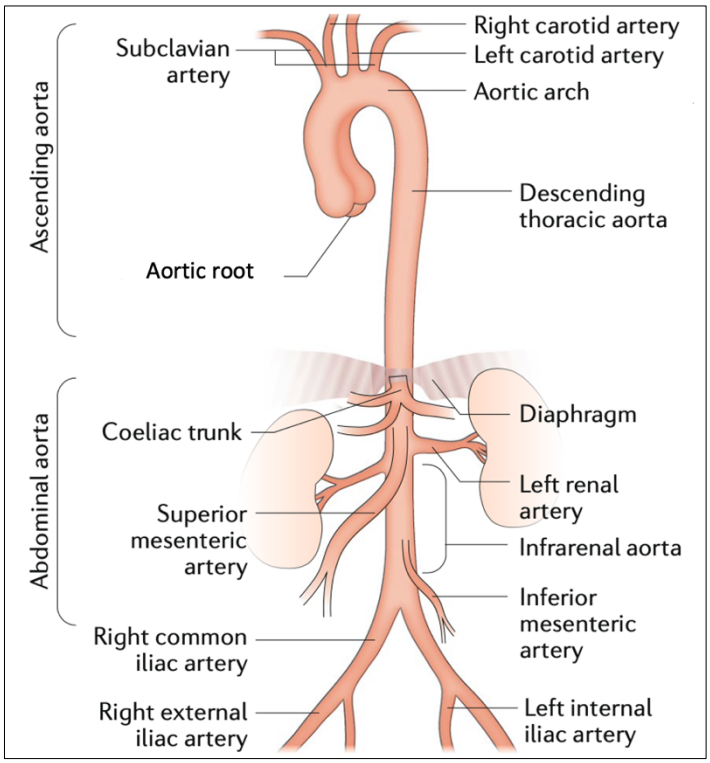

***Figure 2: Aortic anatomy**. Originally from Natzi Sakalihasan et al., 2018*

1.2.3.1 Aneurysms' locations

Aneurysms can develop throughout every blood vessel in the body, but the abdominal aorta is the most frequent site because indeed, thoracic aortic aneurysms (TAA) have a lower reported prevalence than AAAs (Kuzmik et al., 2012; Salameh et al., 2018). The aortic root and/or ascending aorta are the most common places for TAAs with ±60% of prevalence. They are followed by the descending aorta with ±35%, and finally the arch and the thoracoabdominal aorta both at <10% **(Figure 3)** (Mathur et al., 2016). They have a different etiology than AAA. Cystic medial degeneration, which manifests histologically as smooth

muscle cell dropout and elastic fiber degeneration, is the most common cause of ascending thoracic aortic aneurysms (Isselbacher, 2005). This occurs most commonly in later stages of life, around the age of 60 or 70. Aneurysm development is also linked to smoking and high blood pressure (Salameh et al., 2018). Cystic medial degeneration in young individuals has traditionally been linked to Marfan syndrome which results in a decrease of aortic wall's elastin levels weakening its elastic properties, finally leading to dilation and aneurysm development (Isselbacher, 2005; Salameh et al., 2018). Iliac and downstream arteries are also prone to aneurysms. Indeed, a study showed an incidence of 14% of femoral and knee artery, called popliteal artery, aneurysms in patients with AAA (Zipes et al., 2018).

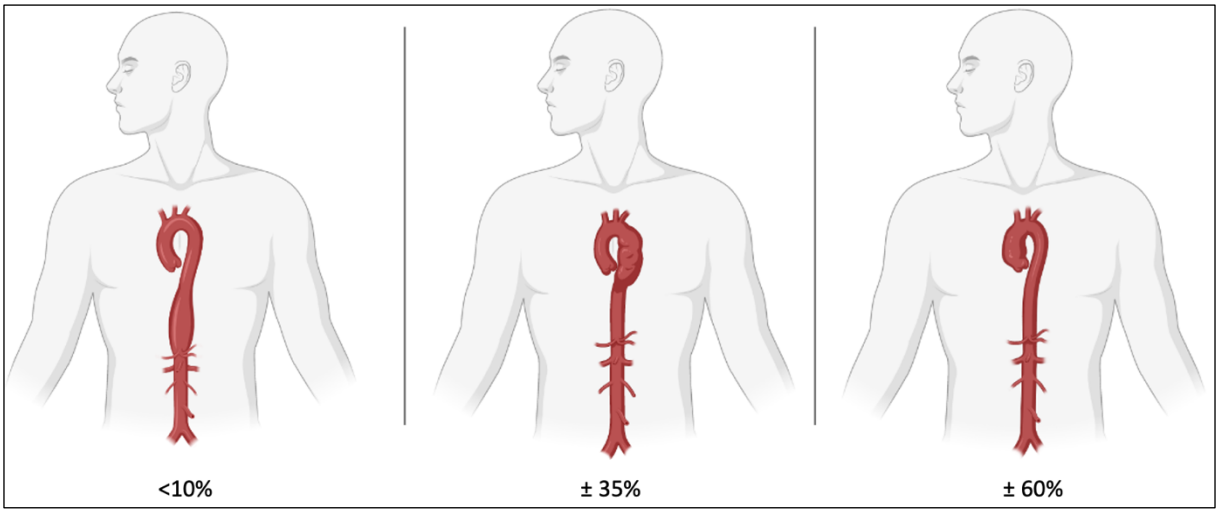

Figure 3: Different locations of thoracic aortic aneurysms. *The thoracoabdominal aorta accounts for less than 10% of TAAs, followed by the descending aorta with more or less 35% and finally the most prevalent type being the ascending aorta with more or less 60%. Designed with BioRender.com.*

1.3 Abdominal aortic aneurysm

1.3.1 Prevalence

A meta-analysis study has been conducted in general population of all continents assessing the prevalence of AAA, which was found to be 4,8% (X. Li et al., 2013). This number is a trend and is different in several studies because most of the time, aneurysms are discovered in their final stage, the rupture. For example, in Sweden, a screening study has shown that the prevalence was 2,2% in a cohort of 65-year-old patients (Svensjö et al., 2011). While in

Norway, in a study including men and women between 25 and 84 years old, a prevalence of 5,3% was found (Singh et al., 2001). Finally, a more recent study in Italy, with men aged between 50 and 75 years old and women between 60 and 75 years old, showed a prevalence of 1,7% (Gianfagna et al., 2018). These studies highlight that the prevalence can vary between countries because of some biases like the different populations studied, the age of the patients, the criteria to join the study and so on.

In England the prevalence of AAA decreased from 1991 to 2015. Indeed, a 25-year longitudinal study, including a large British 65-year-old cohort of men, indicated a decrease in the prevalence of AAA (Oliver-Williams et al., 2018). This might be because of the decrease in the male smokers, smoking being the principal risk factor.

1.3.2 Risk factors

Regarding the risk factors for this disease, the meta-analysis revealed that hypertension, smoking, coronary artery disease, dyslipidemia, respiratory disease, cerebrovascular disease and renal insufficiency were risk factors for AAA in Europe (X. Li et al., 2013). In a Sweden study, the risk factors were hypertension, coronary artery disease and smoking, the latter with the highest odds ratio (Svensjö et al., 2011). The most important modifiable risk factor is smoking (Golledge, 2019). Indeed, the largest study investigating the risk factors contained 3 million individuals and focused more on the smoking status. The effect of smoking on AAA development was higher for smokers, with duration and quantity of packs smoked increasing the risk. However, age remains the most significant risk factor, with the risk increasing starting from 65 years old.

Men were also more at risk than women, while at the same time, among men population, African-American and Asian men were found to be less at risk of developing AAA (Kent et al., 2010). A family history of AAA has been associated with increased incidence of the disease (Salo et al., 1999). According to studies, aged male first-degree relatives have a higher likelihood of developing the condition. These findings point to a genetic component in AAA pathology (Kuivaniemi et al., 2003; Larsson et al., 2009; Sakalihasan et al., 2014; Salo et al., 1999)

Several connective tissue diseases have been associated with AAA. Indeed, patients with uncommon genetic conditions such as vascular Ehlers–Danlos syndrome, Marfan syndrome, and Loeys–Dietz syndrome can develop aortic aneurysm. However, not all aneurysm patients are affected by one of these disorders (Sakalihasan et al., 2018; Shimizu et al., 2006). Therefore, these diseases are not directly associated to AAA pathology.

The 4 most frequent risk factors are therefore older age, smoking, male gender, and positive familial history (Sakalihasan et al., 2018).

Patients with AAAs frequently have atherosclerosis, yet some patients with severe atherosclerosis may not develop AAA (Golledge & Norman, 2010). Although it is unclear if the link between AAA and atherosclerosis is causative or just owing to shared risk factors, but one study showed that a history of atherosclerotic disease was significantly related with AAA (Wanhainen et al., 2005).

1.3.3 Pathophysiology

Since AAA involves all three layers of blood vessels, the major part of the pathophysiology knowledge is on the basis of the histological features and molecular observations of aneurysmal tissues removed during surgery or post-mortem (Bruijn et al., 2021; Gregory T Jones, 2011). Proteolytic fragmentation of the extracellular matrix (ECM), vascular smooth muscle cells (VSMC) apoptosis, chronic immune cell infiltration of the media and adventitia, and increased oxidative stress in the aortic wall are all hallmarks of AAA that will be developed in the following section (Maegdefessel, Dalman, et al., 2014; Sakalihasan et al., 2018).

All these characteristics have been well established, but an increasing number of additional pathways are being implicated in disease. Multiple cellular signaling pathways, such as mitogen-activated protein kinases (MAPK) signaling, nuclear factor-kappa B (NF-kB) signaling, and transforming growth factor beta (TGFß) signaling, are implicated in AAA disorder. Despite this, there is a general lack of knowledge of the function of cellular signaling in disease

progression, since a growing number of new pathways are involved in pathogenesis (Z. Li & Kong, 2020).

AAAs are characterized by the presence of an intraluminal thrombus (ILT) which comprises leukocytes, proinflammatory cytokines, and proteolytic enzymes and is implicated in the genesis, progression, and rupture of AAA (Parr et al., 2011). ILT are multi-layered neo-tissues. Starting from the lumen, there is a blood clot containing circulating elements recruited in the blood stream like inflammatory cells. This part is called the luminal part. And deeper, the abluminal part in direct contact with the vascular wall, characterized by an active fibrinolysis. The size of the ILT is an important factor in the progression of AAA since it thickens the aortic wall. This thickening has a direct effect on the aortic wall by degrading it (Gregory T Jones, 2011; Sakalihasan et al., 2018). Indeed, the ILT induces a functional hypoxia at the intima and media level. This results in inflammation, neovascularization and VSMCs apoptosis. (Shimizu et al., 2006). The proteolytic and oxidative environment present in the aneurysmal tissue contribute to VSMCs apoptosis (Sakalihasan et al., 2018).

The inflammatory cells recruited by the ILT are carried outward through the aortic wall contributing to the ECM degradation. On the other side of the aortic wall, the adventitia's vasa vasorum induce the adventitial immune events, but are also the branches enabling inflammatory cells to reach the media and intima by neoangiogenesis, stimulated by growth factors recruited by the ILT (Sakalihasan et al., 2018; Shimizu et al., 2006).

The dilation and rupture of the aorta are more likely to occur due to ECM degradation that concerns mainly collagen and elastin since they are the components of the matrix that provide the arteries' structural integrity and stability (Shimizu et al., 2006). Activated forms of matrix metalloproteases (MMP) and serine proteases are both enzymes that contribute the most to AAA expansion and have been proved to be present at higher levels in aneurysmal wall compared to normal tissue (Sakalihasan et al., 2018; Shimizu et al., 2006).

1.3.4 Complications

The ultimate and most feared stage of an aneurysm is its rupture. It can cause a potentially fatal intra-abdominal hemorrhage responsible of death in 65% to 85% of the time. The intra-abdominal bleeding due to rupture leads to pain, either abdominal or back depending on the site of rupture, hypotension and a pulsatile mass (Sakalihasan et al., 2018). These three clinical signs represent the classic clinical triad of a ruptured aneurysm. However, only 25–50% of individuals have this triad, and many patients have symptoms and indications that point to a different diagnosis (Assar & Zarins, 2009).

The occurrence of this rupture is mostly due to the asymptomatic aspect of this disease. Indeed, it is diagnosed most of the time incidentally via imaging to explore unrelated abdominal complaints or through AAA ultrasonography screening programs that are available in several developed countries, as exemplified a little above (Golledge, 2019).

Many factors must be taken into account in the context of aneurysm rupture, and the most important and documented one is the growth rate of the aortic diameter. A study to characterize aneurysm growth in a AAA-patients cohort showed a mean growth rate of 2,6 mm/year. Moreover, it was observed that growth was accelerated by 70% in patients who had a larger baseline diameter, and it was 15% to 20% faster in smokers (Brady et al., 2004). These data highlight that the two major rupture risk factors are the diameter of the aneurysm and the smoking status.

1.3.5 Management

Nowadays, the management of patients with a AAA (ruptured or not) is performed by surgery. It can either be an open surgery, performed since 1951, or endovascular aneurysm repair (EVAR) since 1986 (Golledge, 2019; Sakalihasan et al., 2018; United Kingdom EVAR Trial Investigators, Greenhalgh, R. M., Brown, L. C., Powell, J. T., Thompson, S. G., Epstein, D., & Sculpher, 2010). An aortic surgical diameter threshold of 55-mm was established for men and lowered to 50 mm for women since their AAAs are more likely to rupture at lower diameters than men (Buck et al., 2014). Therefore, patients who are incidentally found to have an

aneurysm and under the surgical threshold at the time of an ultrasound examination, are taken in for surveillance. If their aortic diameter becomes greater than the threshold, they are considered suitable for surgery, whether open or endovascular. Elective surgery can also be performed in patients who have a smaller than the threshold diameter, but rapidly growing aneurysm, but also in young and fit patients. On the other hand, in some patients with a diameter greater than the threshold, surgery may not be appropriate due to risk factors, advanced age or comorbidities (Sakalihasan et al., 2018).

1.3.6 Open repair versus endovascular repair

The goal of an open repair surgery is to prevent AAA rupture by inserting an aortic synthetic prosthesis into the aneurysmal sac by dissecting the neck and the distal end of the AAA and replacing it with a graft, mostly made of polytetrafluoroethylene (Golledge, 2019; Sakalihasan et al., 2018). On the other hand, EVAR's goal is to keep the AAA out of the systemic circulation without repairing the injured aorta. It is done with a bifurcated prosthesis inserted via the femoral and iliac arteries (Sakalihasan et al., 2018). The iliac arteries originate at the level of the umbilicus and bifurcate in the legs and continue to give rise to the femoral artery irrigating the leg. The graft is fixed with stents upstream of the aneurysm, at the level of the renal arteries, and downstream, at the level of the iliac arteries, to the walls in the non-aneurysmal aorta, and the aneurysm sac is left in place (Sakalihasan et al., 2018).

A question that was frequently asked was which procedure, either open or endovascular repair, was safer for patients. Clinical trials conducted between 1999 and 2004 could partially answer such question. The first clinical trial comparing EVAR and open repair assessed the 30-day mortality rates. The results showed that patients receiving EVAR had a significantly lower 30-day mortality compared with open repair procedure (Greenhalgh, R. M., Brown, L. C., Kwong, G. P., Powell, J. T., Thompson, S. G., 2004).

Another larger clinical trial called United Kingdom EVAR-1 (UK EVAR-1) assessed the long-term outcomes of endovascular versus open repair of big aneurysms and showed the same results as the EVAR trial. In a follow-up period over 4 years after randomization, they observed no differences in overall mortality or aneurysm-related death but a larger number of reinterventions owing to graft-related complications in the EVAR group, one of them being

endoleaks (Sakalihasan et al., 2018; United Kingdom EVAR Trial Investigators, Greenhalgh, R. M., Brown, L. C., Powell, J. T., Thompson, S. G., Epstein, D., & Sculpher, 2010). Another clinical trial, The Dutch Randomized Endovascular Aneurysm Repair (DREAM) trial, was conducted and corroborated the results above-mentioned (De Bruin et al., 2010).

Based on these findings, a decision must be made for a patient requiring surgery as to the most appropriate surgical approach. The benefits and risks balance must be considered while making a choice. The physician must consider the patient's life expectancy, fitness, anatomic appropriateness that allows for endovascular repair and finally the patient's desire (Moulakakis et al., 2013).

1.3.7 Pharmacological intervention (non-surgical)

Patients who are not eligible for reconstructive surgery have their aneurysm in continuous progression. The best way to stop this progression would be a pharmacological treatment. However, today, there is no proven effective treatment able to slow the growth of AAA and reduce the risk of rupture. Several treatments were extensively tested in animal models. Among these, an inhibitor of the c-Jun N-terminal kinase signaling molecule, from the MAPK pathway, was proven to be efficient in reducing aneurysm's diameter (Golledge, 2019; Z. Li & Kong, 2020; Yoshimura et al., 2017). Statins were considered as good candidates, and have been proved to be responsible for growth rate reduction and a lower aneurysm rupture risk (Salata et al., 2018). But there is the need to advance AAA research in order to provide patients with effective pharmacotherapy in substitution to open repair and EVAR (Yoshimura et al., 2017).

The use of stem cells as a treatment for AAA, like many other diseases, is gaining a lot of interest. Cell therapy has been proposed to prevent AAA growth. In animal models, local infusion of bone marrow-derived mesenchymal stem cells (BM-MSCs), endothelial cells, or VSMCs, as well as systemic injection of BM-MSCs, were demonstrated to be beneficial in slowing the progression of previously formed AAA (Golledge, 2019; Sakalihasan et al., 2018).

Changes in the circulating blood levels of numerous micro-RNAs (miRNAs) have been linked to a variety of cardiovascular diseases (CVDs), indicating that they might be promising therapeutic targets. MiRNAs are interesting targets due to their important role in gene regulation. Hence, their therapeutic potential is now being investigated using two approaches: overexpression through mimicry and inhibition through antagomiRs (Condorelli et al., 2014; Romaine et al., 2015; Zhou et al., 2018). MiR-22, for example, has been shown to decrease myocardial autophagy in mice. As a result, using an anti-miR-22 promoted cardiac autophagy in older animals, preventing post-infarction remodeling and improving cardiac function (Gupta et al., 2016). These therapies, however, have yet to enter human clinical trials. This is beginning to happen in other sectors, which is encouraging. The first candidate to enter clinical trials is miravirsen (SPC3649) for the treatment of chronic hepatitis C virus (HCV) infection. Miravirsen, an antagomiR of miR-122, can bind and hence suppress miR-122, which is required for the stability and proliferation of HCV RNA (Janssen et al., 2013; Romaine et al., 2015).

1.4 Micro-RNAs

1.4.1 Generalities

MiRNAs are small RNA molecules with 19 to 25 nucleotides that act as antisense RNA, adversely regulating their target genes after transcription (Lu & Rothenberg, 2018; Hao Wang et al., 2018). Because miRNAs may be released into circulation in the form of membrane-bound vesicles (exosomes and microvesicles) or in combination with protein complexes, they can control gene expression in the cell where they were synthetized as well as in other nearby or distant cells (Černá et al., 2019). A single miRNA can affect the expression of multiple genes by targeting hundreds of mRNAs. These targets can have a wide range of functions, including transcription factors, secreted factors, receptors, and transporters. Thus, miRNAs have the ability to regulate the expression of one-third of human mRNAs (Esquela-Kerscher & Slack, 2006; Lu & Rothenberg, 2018).

1.4.2 Nomenclature

MiRNAs require a specific nomenclature that gives some information. First, we need to know from which organism a miRNA is coming from. The first part of the nomination gives us the species. Then, we have information about the family of the miRNA, a number that corresponds to the chronology of its discovery. It can be followed by a letter representing potential same family members. And lastly, information about the strand of origin from the stem-loop **(Figure 4).**

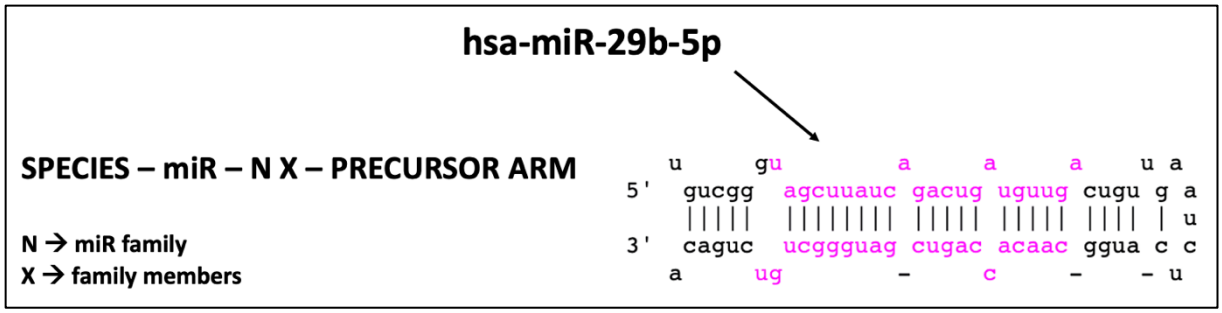

***Figure 4: miRNA nomenclature**. In this example, the miRNA's species is human, 'hsa' standing for Homo Sapiens. It belongs to the 29th discovered family of miRNA. The letter 'b' means that it is related to another miRNA from the same family. And finally, it comes from the 5' arm on the stem loop. Created on PowerPoint®.*

1.4.3 Synthesis and action

The first steps of their synthesis happen in the nucleus. MiRNAs are first synthesized as primary miRNAs or pri-miRNAs, which are longer (over 1 kb) than their mature. They are then cleaved by the RNAase III endonuclease Drosha at the base of the stem-loop structure. The resulting RNA of about 60 to 70 nucleotides is called miRNA precursor (pre-miRNA) (Q. Wu et al., 2012). After being exported to the cytoplasm by the Exportin-5 protein, the pre-miRNA undergoes a second cleavage (Lund et al., 2004). The RNAase III endonuclease, Dicer, identifies the pre-double-stranded miRNA's component and cuts it around two helix turns from the stem-loop structure's base. From this point onward, the loop is cut, resulting in a two-stranded structure. This duplex is made up of two miRNA strands: the strand that will become the final miRNA mature product and the strand that will not. The strand that won't match will be degraded (O'Brien et al., 2018). After their separation by a helicase, only one of the two strands, which is the active form of the miRNA, remains and is inserted into a ribonucleoprotein complex, the RISC (RNA-Induced Silencing Complex). It allows its action on

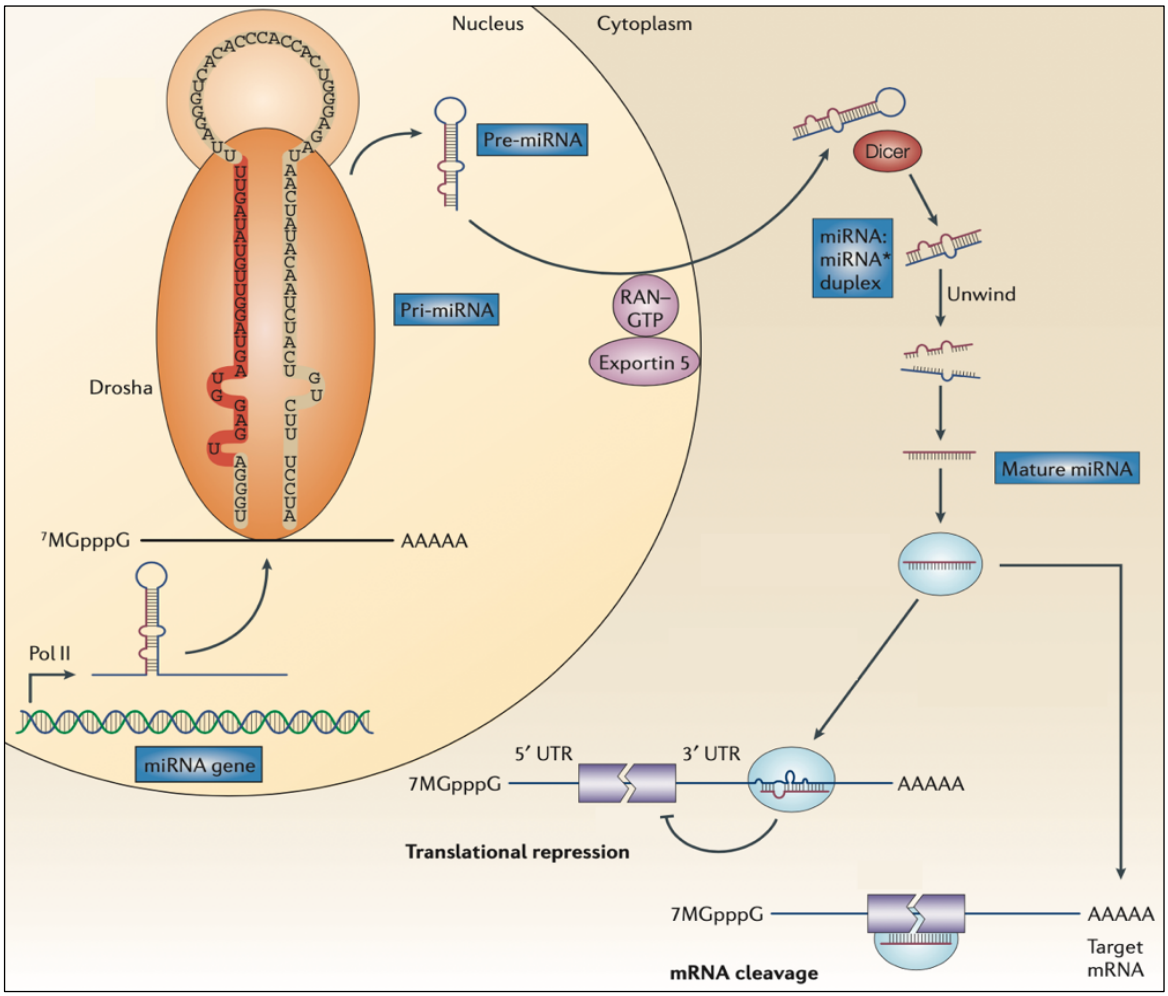

***Figure 5: miRNA biogenesis**. In this example, the miRNA's mature sequence is in red and represents human let-7a-1 miRNA. Adapted from Esquela-Kerscher & Slack, 2006.*

the target mRNAs by a nearly perfect pairing to the target's 3' untranslated region (UTR) through the seed sequence. The seed sequence is the miRNA's sequence comprised between the first 2 to 8 nucleotides that matches perfectly the target mRNA (O'Brien et al., 2018) and triggers its degradation or inhibition of translation (Macfarlane & Murphy, 2010; O'Brien et al., 2018; Q. Wu et al., 2012) (**Figure 5**).

1.4.4 Biomarkers

A "biomarker" can be referred to as a broad spectrum of medical indicators that may be assessed correctly and reproducibly (Strimbu & Tavel, 2010). Biomarkers contribute to our understanding of clinical pharmacology and help us design clinical studies that rapidly and accurately assess the safety and efficacy of a drug. They also provide dosing recommendations and help reduce inter-individual variability in response.

Biomarkers can be used to identify a disease and to monitor health status (*e.g.* blood glucose concentration monitoring for the diagnosis of diabetes), as a tool for disease staging (*e.g.*, malignancies), as an indicators of disease prognosis or for prediction and monitoring of clinical response to an intervention (Atkinson et al., 2001).

1.4.5 Micro-RNAs as biomarkers

What characteristics distinguish a good biomarker? In essence, there are three critical technological characteristics: first the marker must be found in peripheral bodily tissue or fluid (e.g., blood, urine, saliva, breath or cerebrospinal fluid); second, it must be simple to detect or measure in assays that are both inexpensive and reliable; and third, its presence must be linked to tissue damage as precisely as possible, ideally in a quantitative manner ("Biomarkers on a Roll," 2010).

Humans and other animals, such as mice and rats, have miRNAs in their serum and plasma. The levels of miRNAs in serum, also called circulating miRNAs, are uniform, stable, reproducible across individuals of the same species and there is a resistance to degradation by endogenous RNase activity. (Zhou et al., 2018).

Initial screening of circulating miRNAs and development of miRNA signatures from bodily fluids is usually done using microarray profiling, real-time polymerase chain reaction (PCR) array, and next-generation sequencing (NGS) technologies (J. Wang et al., 2016). The serum or plasma miRNA expression profile can be exploited as a novel serum or plasma-based biomarker that could lead to more sensitive and specific testing for disorders with the advancement of precision medicine and NGS (X. Chen et al., 2008; Zhou et al., 2018)

Biomarker-guided diagnoses, treatment, and prognosis are part of personalized medicine, which is becoming an inevitable paradigm in modern cardiology (Adamcova et al., 2018).

1.4.6 Micro-RNA in cardiovascular field

MicroRNAs have been shown to play a role in the formation and function of the heart. This has been proven in a number of gene deletion experiments. Zhao et al. showed that a cardiac deletion of the DICER protein induced pericardial edema and a slowed development of the ventricular myocardium resulting in embryonic mortality owing to heart failure. These findings emphasize the importance of microRNAs in cardiogenesis (Philippen et al., 2015; Zhao et al., 2007). In the adult mouse heart, the DICER protein is also important. Sudden mortality, heart hypertrophy, and ventricular fibrosis were all results of a deletion (Da Costa Martins et al., 2008). These findings show that altering miRNA biogenesis during distinct developmental windows has an influence on embryonic and adult cardiac shape and function (Philippen et al., 2015).

Results from a major sequencing study, as well as other studies, have shown a number of miRNAs that are substantially expressed in non-diseased cardiac tissue and are thus expected to play a crucial role in both normal cardiac function and disease in the healthy adult heart (Romaine et al., 2015).

MiRNAs have been thoroughly established to play a key role in numerous cardiovascular diseases that range from cardiovascular risk factors (such as hypertension, diabetes, and dyslipidemia) through atherosclerosis, myocardial infarction, and cardiac remodeling. They have been proved to regulate smooth muscle cell proliferation and maturation, but also angiogenesis and endothelial cells functions. Since miRNAs are obviously involved in vascular mechanisms, they seem to be related to AAA pathogenesis (Condorelli et al., 2014). Preliminary studies offer tremendous potential for the future use of miRNA as biomarkers and possibly therapeutic targets (L. Ma et al., 2007).

1.4.7 Micro-RNA in cancer

Many human miRNAs seem to be situated in cancer-associated genomic regions or in fragile locations that are subject to mutation, deletion, amplification or translocation in cancer (Bracken et al., 2016). They affect essential processes such as cell proliferation, cell adhesion,

apoptosis, and angiogenesis, the dysregulation of which plays an important role in cancer development, growth, and metastasis. Oncomirs are miRNAs oncogenes that, when overexpressed, trigger cancer. Otherwise, if they slow or inhibit the growth of cancer, they are called tumor suppressors. Because they act on mRNAs, overexpression or underexpression of miRNAs is predicted to result in downregulation or upregulation of the protein product of the target mRNAs, respectively (J. Wang et al., 2016; Yong & Dutta, 2009). Many miRNAs and mRNAs have been shown to be dysregulated in cancer and normal tissues, according to global gene expression patterns (Yong & Dutta, 2009). Indeed, several studies have found altered miRNA profiles in a variety of cancers (J. Wang et al., 2016). They were found not just in blood, but also in serum, plasma, urine, and sputum. Following that, these various miRNAs in these various samples were recommended as biomarkers for various types of cancers, including lung, pancreatic, and prostate cancer, to mention a few (J. Wang et al., 2016).

MiRNAs regulate numerous facets of cancer biology by operating at several levels. They can disrupt the cell cycle by targeting tumor suppressor cell-cycle regulators, for example, and hence cause cancer. They can also target antiapoptotic genes such as Bcl-2, and miR-29b has been implicated in this behavior. As a result, downregulation of these miRNAs by mutation, for example, can result in greater antiapoptotic gene expression and decreased death of tumor cells (Mott et al., 2007; J. Wang et al., 2016).

On the other hand, some miRNAs have been proven to be elevated in several cancers. MiR-21 is one of them. It enhances cell motility and invasion by targeting PTEN (phosphatase and tensin homolog), a tumor suppressor inhibiting cell invasion. It was recently discovered to be implicated in colorectal cancer (Asangani et al., 2007). The other miRNA implicated with metastasis is miR-10b. MiR-10b is increased in metastatic breast cancer cells, likely as a consequence of transcriptional activation by a transcription factor (L. Ma et al., 2007).

To summarize, because miRNAs have the ability to control multiple distinct genomic pathways, their deletion or misexpression is likely to be pleotropic and contribute to illness, including cancer (Esquela-Kerscher & Slack, 2006).

1.4.8 Micro-RNAs in AAA

There is no approved biomarker for AAA illness. Although several probable candidates have been offered, none have been verified in clinic. Among these are plasma MMP-9, D-dimers, and ECM degradation products, all of which arise from or are implicated in matrix degradation, a key feature of AAA (Sakalihasan et al., 2018). Several miRNAs have been shown to be linked to human AAA. Their potential as AAA biomarkers again warrants further exploration (Iyer et al., 2017a).

Several miRNAs have been shown to be expressed differentially in human aneurysmal tissue or blood like miR-21, miR-191-3p, miR-455-3p, miR-1281, miR-29b and miR-155 (Golledge, 2019; Iyer et al., 2017b). MiR-21, for example, has been shown to be increased in nicotine-treated rat models, but it has also been found to be differentially expressed in human aneurysmal tissue compared to healthy tissue (Maegdefessel, Azuma, Toh, Deng, et al., 2012). MiR-29b has also been linked to AAA disease in mouse models, but it was shown to be downregulated in human AAA tissues compared to non-aneurysmal control tissues (Maegdefessel, Azuma, Toh, Merk, et al., 2012). Finally, differential expression of miR-24 in AAA tissue and plasma was reported when compared to controls, and this miR, like the others, might be employed as a biomarker. However, this must be confirmed in broader patient cohorts (Maegdefessel, Spin, et al., 2014).

Although the same miRNAs have been detected in AAA patients and cancer patients, like miR-21 above-mentioned, no research have been conducted to identify a biomarker that is common to both illnesses.

2 Hypothesis, objective, and strategy

2.1 Hypothesis

A study published in The New England Journal of Medicine (De Bruin et al., 2010) has shown that in a cohort of patients with large aneurysms, the proportion of cardiovascular-related deaths after EVAR or open-air surgery was approximately equal to deaths caused by cancer. Based on this epidemiological observation we have hypothesized that there could be a potential link between AAA and cancer. Inflammation has been proven to be an important component in cancer development (Coussens & Werb, 2002) and progression as well as in AAA pathogenesis (Hellenthal et al., 2009). The question would be to investigate whether AAA is inducing cancer or *vice versa*. Since AAA involves chronic inflammation of the aortic wall, we hypothesize that the aneurysmal tissue would release in the circulation inflammatory markers, but also other molecules like nucleic acids like miRNAs, and that these released molecules may have a systemic effect and could be able to induce and/or exacerbate cancer.
To verify this hypothesis, the hosting laboratory of Professor Sakalihasan has been collecting data from a cohort of 103 AAA patients for ten years, between 2008 and 2018. In this population, 34 patients developed cancer and 69 did not. Among these cancers, there were pulmonary cancers, accounting for one third, urologic cancer for one third, and the rest included mammary, blood and digestive cancers. Statistical analysis in such cohort has proved AAA to be a risk factor for cancer when correcting for smoking and age.
This motivated us to search for candidate biomarker(s) in this cohort. As mentioned in the introduction, miRNAs have been proven to be good candidates as biomarkers since they are stable in the circulation.

2.2 Objective and strategy

Our objective is to prove that there is a link between AAA and cancer by determining the miRNAs that are differentially expressed in AAA/cancer patients versus AAA patients without cancer and that can be used as predictive tool.

To do so, we extracted RNA from 5 AAA patients that developed cancer and 5 AAA patients that were not affected by cancer during the 10 years follow-up.

The RNAs have been sequenced at the GIGA-Genomics platform by NGS. Based on these results, we selected two target miRNAs that we validated by quantitative reverse transcription PCR (RT-qPCR) on the 10 patients as well as on 20 selected patients from the cohort.

3 Material and methods

3.1 Human plasma collection and preparation

Approval for studies on human samples was obtained under informed consent according to the ethical file (Belgian Nr : B70720095967; Ref.: 2009/54). 103 AAA patients were recruited from the University Hospital of Liège, Belgium, between 2008 and 2011, and followed-up until 2018. Plasma was isolated and several 100 µl aliquots were made and stored at -80°C until use. More recent plasma samples were collected on healthy people, using same procedures.

3.1.1 Plasma Isolation from healthy donors

Whole blood (3 ml) was added to an ethylenediamine tetraacetic acid (EDTA) anticoagulant tube, and then centrifuged at 3 000g for 10 minutes at room temperature (RT). Several aliquots of 110 and 220µl were made and stored at - 80°C.
This plasma is used to set up the procedures of RNA isolation and RT-qPCR.

3.2 Total RNA isolation

Total RNA from 10 patients was isolated using the miRNeasy® Mini Kit 50 #Cat. No. 217004 (Qiagen, Germany). 110µl of plasma were added to 550µl of QIAzol #Cat. No 79306 (Qiagen, Germany) , mixed and incubated 5 minutes at RT. 110µl of chloroform from Normapur® #Cat. No. 22711.324 (VWR, United States of America) were added, mixed again, and incubated 3 minutes at RT. Following centrifugation at 12 000g for 15 minutes at 4°C, 300µl of the supernatant were collected and 1,5 volumes of absolute ethanol from Normapur® #Cat. No. 20821.321 (VWR, United States of America) were added, and the Eppendorf was mixed by inversion. 700µl of the mixture were put in a RNeasy Mini spin column with a collection tube and centrifuged at maximum speed (17 000g) at RT. 700µl of RWT buffer, 500µl of RPE buffer and 500µl of ethanol 80% from Normapur® #Cat. No. 20821.321 (VWR, United States of America) were successively added with a brief centrifugation between each step. The column was put in a new collection tube and centrifuged 2 minutes at maximum speed (17 000g) at RT. The column was again put in another collection tube and centrifuged 5 minutes at full speed with the lid open to dry the membrane. The column was put in a 1,5ml Eppendorf and

25µl of RNAse-free water were added directly onto the RNeasy mini column membrane and eluted by centrifugation at ≥ 8 000g for 1 minute. The eluate was taken on the membrane again for a second elution to increase the yield. The RNA concentration was assessed using Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific, United States of America).

3.3 Sequencing

The 10 isolated total RNAs were sent to the GIGA-Genomics platform from the University of Liège to perform a NGS of miRNAs. Raw data was in the form of FastQ files and quality control (QC) in form of FastQC. We have uploaded the data to the GeneGlobe's complimentary web-based analysis pipelines (Qiagen, Germany) to run the analyses. The GeneGlobe Data Analysis Center contains a number of web-based tools to help optimizing and simplifying data analysis.

3.4 GeneGlobe's analysis pipeline

We ran the analysis and got a volcano-plot plotting significance and fold change of miRNAs. We also got a file with all the listed miRNAs, their fold change value, p-value, raw counts, and normalized counts. Our goal was to validate the sequencing results with qPCR. We decided then to choose 2 target miRNAs based on 2 criteria; their normalized counts and a p-value set under 0,11. We made sure that target we chose were the ones with the higher normalized read counts to increase our chances to find copies amplified during qPCR.

3.5 RT-qPCR

To validate the sequencing results, we reverse transcribed our RNAs into cDNAs and then performed a quantitative PCR. The primers used in the qPCR are Locked Nucleic Acids (LNA) oligonucleotides from Qiagen. This technology has several benefits compared to classic primers. When compared to typical DNA or RNA oligonucleotides, they exhibit far higher affinity for their complementary strand. This results in remarkable sensitivity and specificity, making LNA-enhanced oligos great instruments for identifying and discriminating tiny or extremely identical DNA or RNA targets in a wide range of scientific applications.

First, we used miRCURY LNA RT Kit #Cat. No. 339340 (Qiagen, Germany). 4µl of RNA was used in the mix for the reaction along with 4µl of reaction buffer containing the RT primers, 9µl of

RNAse-free water and 2µl of enzyme mix containing both the poly(A) polymerase and the reverse transcriptase. The reaction mixtures were incubated 60 minutes at 40°C and then 5 minutes at 95°C to heat inactivate the reverse transcriptase. The cDNAs were kept overnight at 4°C before qPCR.

Before starting with the mixtures, the primers from miRCURY LNA miRNA PCR Assays kit #Cat. No. 339306 (Qiagen, Germany) are delivered in a dry form and needed to be resuspended in 220µl of RNAse-free water from Invitrogen #Cat. No. 15667708 (Thermo Fisher Scientific, United States of America) and left 20 minutes at RT. Information about the primers used are listed in **Table 1**. To optimize the amount of cDNA to use in the reaction and to determine the efficiency of each couple of primers we have used undiluted sample, 1:10, 1:100 and 1:1000 dilution. To further optimize the dilution of cDNA to use for each patient, we conducted qPCR with 1:10, 1:30 and 1:100 cDNA dilution. We subsequently used a 1:10 cDNA dilution for all downstream qPCR reactions.

In a total reaction volume of 10µl, we added 5µL of SYBR green master mix #Cat. No. 06924204001 (Roche, Switzerland) with 1µl of the resuspended PCR primer mix and 1µl of water, to which we added 3µl of cDNA template. All PCR reactions were run in duplicates with LC480

The results of PCR reactions were calculated using the $2^{-\Delta\Delta CT}$ method. The delta-delta Ct method, also known as the $2^{-\Delta\Delta CT}$ method, is a simple formula used to calculate the relative fold gene expression of samples when performing RT-qPCR.

Name	Accession in miRBase	GeneGlobe ID	Mature sequence
hsa-miR-142-3p	MI0000458	YP00204291	5'- UGUAGUGUUUCCUACUUUAUGGA-3'
hsa-miR-122-5p	MI0000442	YP00205664	5'-UGGAGUGUGACAAUGGUGUUUG-3'
hsa-miR-374a-5p	MIMAT0000727	YP00204758	5'-UUAUAAUACAACCUGAUAAGUG-3'

Table 1: list of LNA primers for qPCR

3.6 Statistical analysis

Mann-Whitney U test was done using GraphPad Prism 9.3.1.

4 Results

4.1 NGS profiling

We decided to perform a genomic profiling of 8 plasma samples through NGS, after eliminating 2 plasma samples from the initial cohort, in order to identify miRNAs differentially expressed between the cancer and non-cancer group of patients who developed AAA. Our cohort is composed of men aged between 52 and 86 years old. All patients were, or were former smokers, except for 2. The miRNAs available in the results have been matched with the ones listed on miRbase. The miRBase database contains searchable records for published miRNA sequences and annotations. The volcano-plot shows us that out of 2632 miRNAs annotated with miRbase, 46 were statistically significant, with a p-value threshold set at 0,11. Seventeen had a positive fold change and 29 had a negative fold change. Our two target miRNAs are hsa-miR-122-5p and hsa-miR-142-3p with a p-value of 0,10 and 0,06 respectively, and a fold-change of 1,90 and 1,91 respectively (**Figure 6**).

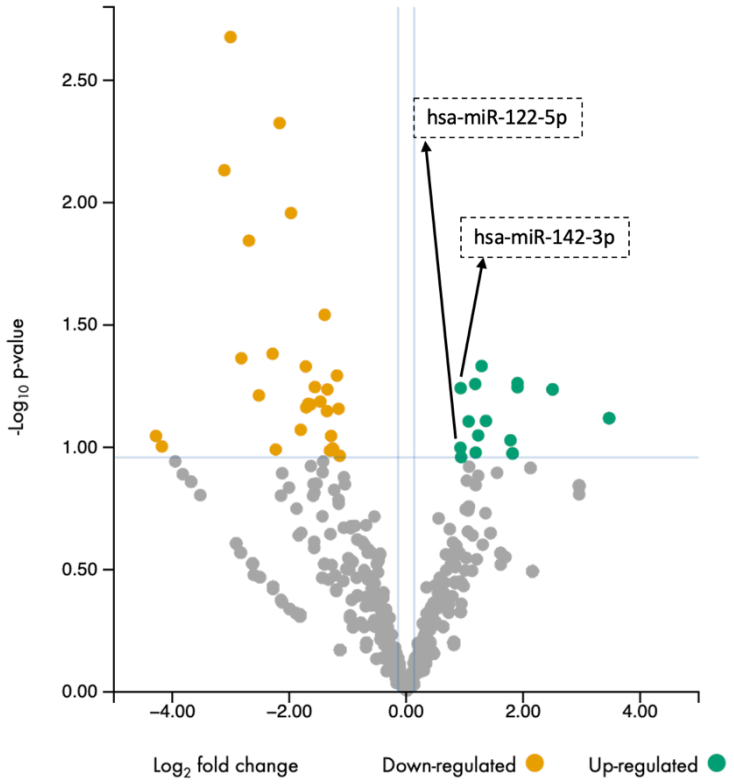

***Figure 6**: Volcano plot of differentially expressed plasma-derived micro-RNAs of AAA patients that developed cancer within 10 years and that did not develop cancer (fold change]-1;1[, p-value ≤ 0,11).*

4.2 Validation of sequencing results by RT-qPCR

As shown by the sequencing results, hsa-miR-122-5p and hsa-miR-142-3p met our criteria to be selected as targets. Hence, we decided to validate by RT-qPCR this result on the same 10 patients that we sequenced and then on 20 more patients. The GeneGlobe's complimentary web-based analysis pipeline not only determines up or downregulated miRNAs, but also normalization miRNAs (miRNAs equally expressed in both groups). Among them, the software proposed hsa-miR-374a-5p as miRNA to use for relative quantification of our target miRNAs.

4.2.1 hsa-miR-122-5p

In the first instance, to validate the sequencing results, we did a RT-qPCR on the 10 patients that were sequenced. We found an 8,604 to 1,747 ratio of $2^{-\Delta\Delta CT}$ median values for cancer and non-cancer group respectively, corresponding to a fold change value of 4,92 (**Figure 7**). This fold change is higher for qPCR than for NGS.

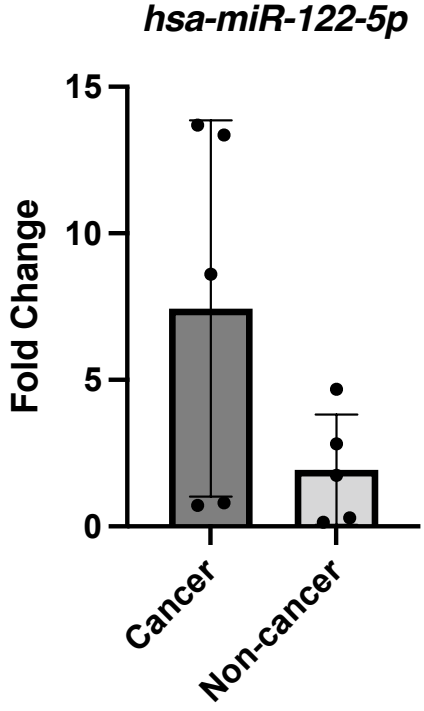

***Figure 7**: Differential expression of hsa-miR-122-5p in cancer versus non cancer patients. Cancer patients (n=5) show an up-regulated value of miRNA compared to non-cancer group (n=5) samples. Data shown as median with 95% confidence interval (CI).*

Then we have extended the validation to more patients of the cohort. During qPCR, miRNAs of 5 patients were undetectable. Therefore, the final validation was done on 25 patients. Here, the ratio is 4,6 to 1,1 for cancer and non-cancer group respectively, corresponding to a significant fold change value of 4,05 (**Figure 8**).

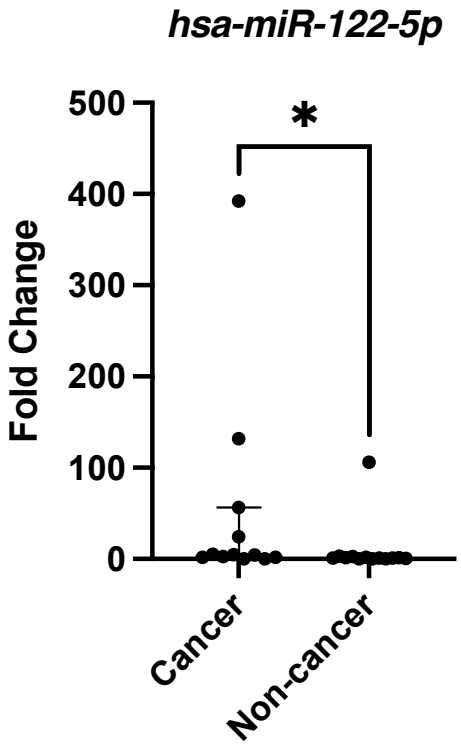

***Figure 8**: Differential expression of hsa-miR-122-5p in cancer versus non cancer patients. qPCR results show that hsa-miR-122-5p cancer group (n=12) samples are significantly up-regulated compared to non-cancer group (n=13) samples for the whole cohort. Data shown as median with 95% confidence interval (CI).*

4.2.2 hsa-miR-142-3p

Again, we aimed to validate the sequencing results by qPCR on the 10 first patients for this miRNA. Again, the fold change is positive with a 1,685 to 1,135 ratio for the cancer and non-cancer group respectively, resulting in fold change value of 1,48 (**Figure 9**) This time, the fold change value from the NGS is lower than the qPCR one, but still positive.

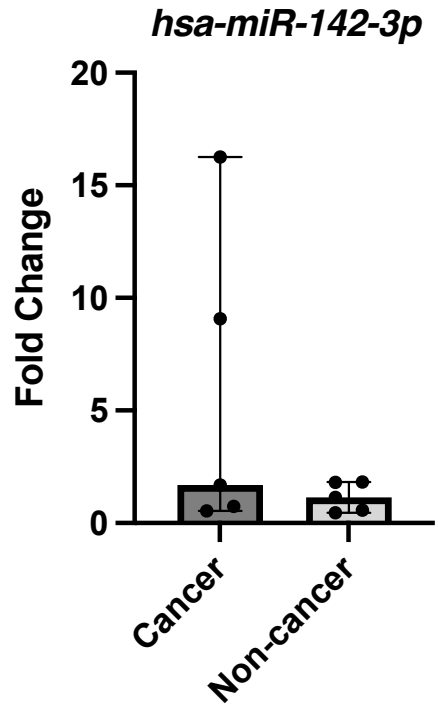

***Figure 9**: Differential expression of hsa-miR-142-3p in cancer versus non cancer patients. Cancer group (n=5) samples are up-regulated compared to non-cancer group (n=5) samples for 10 patients sent for NGS. Data shown as median with 95% confidence interval (CI).*

This time, 2 patients had undetectable levels of miRNA during qPCR. qPCR was therefore done on 28 patients. The results show a positive fold change with a 2,650 to 0,6482 ratio for the cancer and non-cancer group respectively, corresponding to a non-significant 4,09-fold increase (**Figure 10**).

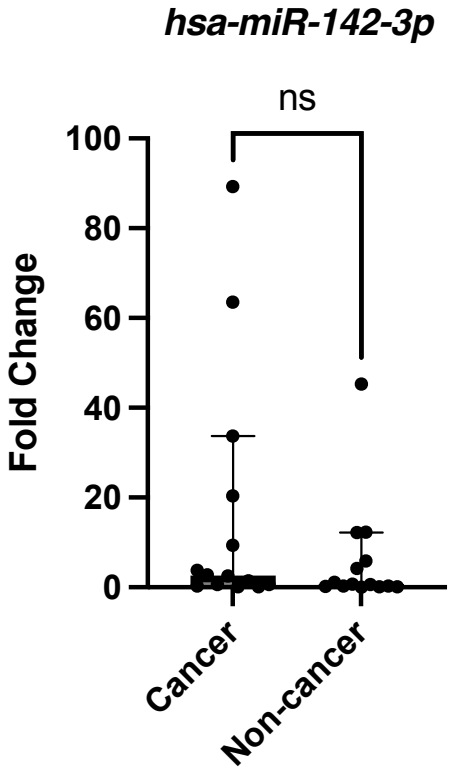

***Figure 10**: Differential expression of hsa-miR-142-3p in cancer versus non cancer patients. qPCR results show that hsa-miR-142-3p cancer group (n=14) samples are non-significantly up-regulated compared to non-cancer group (n=14) samples for the whole cohort. Data shown as median with 95% confidence interval (CI).*

5 Discussion

MiRNAs in circulation are homogenous and stable among individuals of the same species. Microarray profiling, PCR array, and NGS techniques were used to screen for circulating miRNAs and establish miRNA signatures from physiological fluids. With the progress of precision medicine and NGS, the serum or plasma miRNA expression profile can be used as a new serum or plasma-based biomarker, potentially leading to more sensitive and specific testing for diseases.

Cancer and cardiovascular diseases have been shown to be both responsible of death at the same extent in a cohort of patients who received a repair surgery of abdominal aortic aneurysm (De Bruin et al., 2010). Therefore, on a cohort of AAA patients with both cancer conditions, we decided to study the potential link between cancer and this cardiovascular disease by looking for a miRNA as a biomarker. By NGS followed by RT-qPCR, we reported that hsa-miR-122-5p was significantly upregulated in the cancer patients.

MiR-122-5p has been proved to be involved in many cancers like renal cell carcinoma (Heinemann et al., 2018), gastric cancer (Xu et al., 2018), hepatocellular carcinoma (J. Ma et al., 2018), pancreatic ductal adenocarcinoma (Dai et al., 2020) and non-small cell lung cancer (NSCLC) (X. Wu & Fan, 2019). Clinical and experimental evidence strongly supports miR-122's physiological and pathophysiological functions in cardiovascular fibrosis and dysfunction. Its overexpression appears to be a direct player in the deterioration of the cardiovascular system in many cardiovascular disorders by inducing inflammation, oxidative stress, apoptosis, and ECM deposition (Y. Liu et al., 2020). As for cancer, this miRNA has been associated with several diseases and assigned a biomarker function. Among them, hypertension, acute myocardial infarction, heart failure and atherosclerosis (Stojkovic et al., 2020; Y. L. Wang & Yu, 2018; Yu Wang et al., 2018; Zhang et al., 2020). We showed that miR-122-5p was overexpressed in patients with AAA who developed cancer (pulmonary, urologic, and digestive), which is confirmed by all these studies. As this miRNA is differentially expressed between the two

conditions, these findings suggest that this miRNA may be a potential candidate biomarker for both AAA and cancer.

Regarding the other miRNA miR-142-3p, results were different. It is also overexpressed in cancer patients, but not significantly. This miRNA, like the above-mentioned, is either associated or potential biomarker to many cancers. Among these are the following non-exhaustive malignancies like colorectal carcinoma, lung adenocarcinoma, NSCLC and gastric cancer (Gao et al., 2019; Ghanbari et al., 2015; Y. L. Wang & Yu, 2018; Yi Wang et al., 2018).
Unlike miR-122-5p, this miRNA has not been established as a biomarker for cardiovascular disease, although it has been linked to inflammatory disorders such as atherosclerosis, heart transplantation, sepsis, and systemic lupus erythematosus (Qin et al., 2018; Van Huyen et al., 2014; Yilun Wang et al., 2016; Yuan et al., 2016).
AAA is a chronic inflammation of the aortic wall due to a massive infiltration of inflammatory cells. It is therefore not inappropriate to have looked for this microRNA in the patients' plasma, but at the same time it is not optimal because it is not specific to both diseases as highlighted by the results. Furthermore, even though it has been showed in inflammatory context, it still has implication in the cardiovascular system at the embryonic level as shown in the zebrafish (Z. Y. Chen et al., 2017; Nishiyama et al., 2012).

Our work can be divided into 3 steps: RNA isolation from plasma, sequencing, and validation by RT-qPCR. All these steps have their limitations and always need to be optimized in order to minimize these.

There is still discussion on whether serum or plasma is the best sample type for detecting both miRNA and RNA expression. Several studies have indicated that plasma samples had a better recovery of miRNA (based on real-time RT-PCR), whereas others have found no or very little difference between serum and plasma (Kroh et al., 2010; Mcdonald et al., 2011; Nishiyama et al., 2012). Based on this, we decided to work on plasma samples.

RNA is present in plasma, meaning that its quality is depending on several pre-analytical variables like sample collection and storage. In our case, information on these two variables was not available due the long-term storage of these samples.

For numerous reasons, we chose sequencing over other more traditional approaches for our methodology. Microarrays require an excessively high quantity of RNA input for biofluid samples, and the sensitivity range is limited. Because of its high sensitivity and specificity, qPCR is a common discovery method. However, the targets must be identified beforehand to capture all sequences of a particular size and quality, while NGS does not necessarily require knowledge of the different miRNAs present in the samples of interest. NGS provides a method for profiling the complete miRNome and detecting new miRNAs and other types of short RNAs that might be biomarkers. Furthermore, it enables profiling of low-input samples like liquid biopsies. As a result, NGS has emerged as the discovery technique of choice for screening.

The library preparation is critical to the success of NGS process. This procedure prepares DNA or RNA samples to be compatible with the sequencer. Our sequencing libraries were produced by fragmenting cDNA and attaching specific adapters to both ends. These adapters contain complementary sequences that allow the cDNA fragments to attach to the flow cell. After that, fragments may be amplified and purified. Molecular index sequences, or "barcodes," are used and inserted to each library during adaptor ligation. New miRNA NGS library preparation approaches based on unique molecular indices or identifiers (UMI) used in our work eliminate quantification bias (Fu et al., 2018). During data processing, these barcodes are utilized to differentiate across libraries (Illumina, 2022; Qiagen, 2019a).

The last step before data analysis is the cluster creation, a process that amplifies clusters of DNA fragments, resulting in millions of clones of single-stranded DNA. Modified nucleotides attach to the DNA template strand in a process known as sequencing by synthesis. The fluorescent signal identifies which nucleotide was added, and the terminator is cleaved to allow the next base to attach (Illumina, 2022).

For our sequencing library preparation, a minimum of 200 pg/µl was needed. 3 out of the 10 isolated RNAs were in the range of concentrations. For the other, they were too low. We decided to proceed with the sequencing, nonetheless. A study showed that if UMIs are incorporated, little PCR bias is produced during small RNA library synthesis (Wong et al., 2019).

The sequencing data we got from the 10 patients were treated with GeneGlobe's complimentary web-based analysis pipelines (Qiagen, Germany). This enabled us to check on the QC results of our samples. Based on these, out of the 10 patients, 2 had outliers' reads values, thus we decided to exclude them for the analysis. Traditional miRNA library sequencing yields raw read counts that frequently reflect PCR bias, and the number of reads does not precisely represent the number of copies of the original RNA. Thus, the target miRNAs cannot be chosen on this basis.

Our selection was based on the counts per million (CPM), which are the normalized reads. Because the sequencing depth, or the number of times a specific nucleotide in the genome is read, might vary between samples, a per-sample library size normalization must be done before samples can be compared (CLCbio, 2019).

Library size normalization is accomplished automatically for the RNA-Seq tools that compare samples (differential expression for RNA-Seq) using the TMM (trimmed mean of M values) approach, a biostatistics method developed by Robinson et al. (Robinson & Oshlack, 2010).

RT-qPCR is a quick, very sensitive, and low-cost technology that provides linear detection over a wide range of orders of magnitude. Furthermore, most labs' setup and data analysis activities are quite easy. It also takes little input RNA, which is useful for biofluid samples, which are frequently poor. qPCR may be adapted to handle several research demands, from screening and profiling to NGS study validation and functional study verification. In our case, we used it to validate our NGS results.

Our methodology was to first validate on the same patients we sent for sequencing. The results were corroborated; the cancer group had higher expression for both miRNAs. From there on, we extracted new RNAs to validate on new patients from the cohort. This brings limitations for the interpretation of our results. Repeatability is an important parameter in scientific experiment. It can be referred to as agreement between successive results obtained with the same method on an identical material submitted to the test under the same conditions, meaning same operator, same measuring apparatus, same laboratory and repetitions on a short duration (Florent, 2017). The fact that we first validated on the patients

who underwent NGS and then on new patients of the cohort introduces a bias on the timing. Indeed, RNA isolation was done in two different times (first batch of isolation for the 10 samples sent for sequencing and the second batch for the rest of the samples). This parameter could explain the differences in fold changes and p-values between NGS and qPCR results. Using different plates must also have introduced bias in our experiment. Indeed, to be sure of the efficiency of PCR, we should have done a standard curve on each plate or add a positive control, thing that we did not do.

qPCR experiments require a normalization gene. In our case, the analysis pipeline gave us a list of normalization miRNAs based on their fold change value of more or less 1. We selected 2 miRNAs for normalization, hsa-miR-128-3p and hsa-miR-374a-5p. A qPCR validation allowed to select hsa-miR-374a-5p at the detriment of the other one being less constantly expressed in all samples.
In the literature, hsa-miR-16-5p has been used in many studies as endogenous reference gene (Lange et al., 2017; Rinnerthaler et al., 2015; Song et al., 2012) and we found it highly expressed, in terms of CPM, in all sequenced patients, but surprisingly it was not suggested as a reference gene by the Qiagen pipeline. Other reference gene candidates are suggested by Qiagen (Germany) for their RT-qPCR kit : miR-103a-3p, miR-191-5p, miR-423-5p, miR-16-5p, miR-425-5p, miR-93-5p and miR-451a (Qiagen, 2019b).

Furthermore, synthetic miRNA could be employed as spike-in controls in challenging samples such as plasma. Spike-ins, also known as exogenous controls, are synthetic RNA oligonucleotides that are introduced to the sample at a known amount to monitor extraction efficiency or sample input amount (ThermoFisher, 2016).
The RT-qPCR kit does not specify the amount of RNA to be used in the RT reaction. This introduces a huge bias for the qPCR since all the patients do not have the same RNA concentration. Indeed, the non-cancer patients had lower RNA concentrations than cancer group after the RNA isolation (data not shown). Meaning that for the qPCR, the results might differ between patients due to the initial differences in RNA concentration.

Based on the literature, some miRNAs, miR-21, miR-24 and miR-29b, have been proved to be involved in AAA pathology in mice and humans (Maegdefessel, Azuma, Toh, Deng, et al., 2012;

Maegdefessel, Azuma, Toh, Merk, et al., 2012; Maegdefessel, Spin, et al., 2014) and in different cancers (Capodanno et al., 2013; Z. Liu et al., 2018; Hongyan Wang et al., 2015). We expected seeing them in our NGS profiling. They were present, but not prominently. It is probable that increasing the number of patients sequenced will result in these miRNAs being more expressed than in our cohort of 10 patients.

Finally, our database includes patients with 4 different types of cancers, namely urologic, pulmonary, digestive, and other various cancers in smaller proportion. This means that the miRNAs that we found more elevated are not specific to one type of cancer but more to the malignancy status of the cancer. It would be better to make the same experiment focusing on one specific cancer to allow the potential discovery of a more cancer-specific biomarker.

6 Conclusion and perspectives

To conclude, through this work, we wanted to highlight a differential expression of miRNAs between cancer and non-cancer patients with a background of AAA. Our results showed that hsa-miR-122-5p was significantly upregulated in cancer group. This miRNA could therefore be considered as a potential candidate predictive biomarker for malignancy in AAA patients.

Despite the lack of defined methods for the use of miRNAs in present clinical practice, there is intriguing evidence that they are a reliable tool for future usage. These molecules satisfy most of the criteria for an excellent biomarker, including accessibility, high specificity, and sensitivity. Despite current limitations, miRNAs as biomarkers for many diseases continue to be an exciting study subject. As existing methodologies advance, miRNAs could become a standard strategy in the generation of individualized patient profiles, allowing for more targeted treatment interventions.

In the future, the sequencing part of this project should be done on the whole cohort of 96 patients to have more statistically significant results and maybe highlight another miRNA with more stringent p-value conditions. It could be interesting to go beyond the RT-qPCR validation. Indeed, in situ hybridization could be done on aortic tissues to detect the presence of miRNA on the tissue directly. This could help answer a part of our hypothesis i.e., to determine if, indeed, the miRNAs found in the circulation originate from the aneurysmal tissue. Immunohistochemical analysis could also be done on tissues to look for proteins regulated by a specific miRNA.

If this miRNA is confirmed having a role in both pathologies, it could be interesting to work on a bigger cohort with different ethnic groups, in order to reduce the genetic polymorphism bias.

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