‘STUDY OF MOLECULAR MECHANISMS INVOLVED IN CARDIOVASCULAR ALTERATIONS IN MARFAN SYNDROME TO SUGGEST A NEW PHARMACOLOGICAL STRATEGY TO IMPROVE PATIENT’S LIFE EXPECTANCY’

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‘Study of molecular mechanisms involved in cardiovascular alterations in Marfan Syndrome to suggest a new pharmacological strategy to improve patient’s life expectancy’

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ABSTRACT

Marfan Syndrome is a connective tissue disease originated by mutations in the FBN1 gene. Aortic aneurism is the most severe consequence of this syndrome and the most common cause of death in MFS patients. Many factors are altered in the molecular mechanisms involved in MFS’s cardiovascular affectations, such as TGF - β, NO, COX-1, COX-2, MMPs (2 and 9) … Since the cure for MFS remains unknown, a treatment based on Nanoparticles loaded with MMP inhibitor and conjugated with elastin ab is proposed as new pharmacological strategy to improve patient’s life expectancy.

INTRODUCTION

Marfan Syndrome (**MFS**) is an autosomal dominant inherited connective tissue disease caused by a mutation in Fibrillin gene 1 (Fbn-1) which can lead to various skeletal, pulmonary, ocular, and cardiovascular system disorders (1). High variability has been documented, and, interestingly, we can even find clinical variation within the same family (same genetic background) and, theoretically, there is no gender differences.

Being able to elaborate a correct procedure to diagnose in an effective way those people with MFS has always concerned clinicians. In order to do so, it is really important to know which are those characteristics that can confirm the MFS diagnostic. According to The Berlin Criteria published in 1986 (2), MFS was detected just by phenotypic features. Later on, a connection between MFS and FBN1, a gene that codifies for a protein called Fibrillin, was discovered (3). Since then, in 1996, another diagnosis criteria appeared, the Ghent Criteria (Ghent-1), based on a revision of the Berlin Criteria but including mutations in FBN1 as a complement for the determination. Finally, in 2010, by an update of Ghent-1, Ghent-Nosology- II was stablished as the actual criteria to diagnose Marfan Syndrome that included, above all, not only mutations in FBN1, but specific characteristics such as ectopis lentis and aortic dilation (4).

According to the results obtained from a study of the Danish population based on Ghent Nosology II diagnosis criteria it affects 6,5/100.000 people (5). It is relevant to mention the importance of the moment of diagnostic, considering that an early detection, can improve the prognostic of the patient, mainly with the consequences related to aortic dilation. Nowadays, the average age of MFS diagnosis is 19 years.

MFS phenotypical characteristics are tall and thin people with disproportionately long limbs. Furthermore, there are specific features such as aortic dilation (evaluated through Z-Score), pectus excavatum, deteriorated teeth, ectopis lentis, positive Steinberg Test, high palate, skin striae distensae, arachnodactyly, lens dislocation and myopia. Life expectancy of MFS patients depends of the severity of

cardiovascular affectation, although it has significantly improved over the last 30 years, thanks to medical and surgical advances (6)

MATERIAL AND METHODS

Objectives

Firstly, main objectives were stablished as a guideline to elaborate the review:

- Assimilate general characteristics of MFS; phenotypic and genotypic.
- Fully understand the different affected cardiovascular molecular mechanism in MFS
- Research on the current treatment for MFS.
- Being aware of the latest clinical trials on MFS treatment strategies.
- Suggest a new pharmacological strategy to improve patient's life expectancy, considering the molecular mechanisms involved in MFS.

Bibliographical research

To fulfill such objectives several databases had to be examined.

Starting with Pubmed, over 50 articles were consulted following strict search criteria. Depending on what was intended the information being searched for, the database was first stablished (Gene, Protein, Nucleotide, …). Then, release and revision date were set to obtain articles published over the last decade. Furthermore, after knowing that G. Egea is probably one of the most well-known investigators in MFS research, search was focused on finding his most recent publications on the field. Directly from his articles, many references were checked and revised to obtain more specific information mainly about molecular mechanisms involved in MFS in different situations.

To collect information about Fibrillin-1 protein and other proteins involved in MFS pathology, protein databases were consulted, Uniprot proving to be the most useful.

Detailed information about FBN-1 gene was gathered basically from Ensemble and OMIM.

To get to know which current MFS investigation and clinical trials are currently taking place, clinicaltrials.org was consulted and used to get a clear idea about which direction MFS treatments are taking.

Finally, a brief interview took place with Dr. Evangelista (Director of the Multidisciplinary Unit of MFS in Catalunya and Coordinator of the Aortic Pathological Unit of Vall d'Hebron Hospital) who is a reference in treating MFS patients and research.

GENETIC BACKGROUND

FBN1

Gene	FIBRILLIN-1; FBN1
Organism	*Homo Sapiens (Human)*
Location	15q21.1
Exon count	66
Size	237483 bp

Table 1. General information on FBN1.

Fibrilin-1 is a 330 kDa glycoprotein formed by 57-59 domains; 47 EGF-like (43 calcium-binding EGF), 8 TGF-β binding (TB) and 2 hybrid domains (EGF-like + TB) which serves as a structural element of calcium-binding microfibrils. These microfibrils are responsible for structural support to elastic and non-elastic connective tissues (7).

Mutations in FBN1 are the genetic basis of MFS. FBN1 is a gene that codifies for a protein Fibrilin-1. Firstly, it generates a preprotein which is processed proteolytically to obtain two different proteins, one of them is involved in

Extracellular Matrix organization and deposition (8).

Once protein Fibrilin-1 has been synthetized, it is transported towards the extracellular matrix due to its structural function. When Fibrilin-1 gets to the EC matrix they bind between them to form microfibrils. Then, binding of microfibrils result in the formation of elastic fibers which allow skin, ligaments and blood vessels to stretch. These fibers also play an important role in supporting functions in more rigid tissues, such as bones, nerves and the eye's lens (9).

In addition, microfibrils are responsible for TGF- β regulation by its binding and avoiding its interaction with its targets. This factor, is involved in growth and cellular proliferation control, differentiation, motility and even apoptosis. Therefore, the aforementioned microfibrils have the capacity to inactivate this factor by its binding, and consequently, activating it by its releasement (10).

In MFS, more than 1300 mutations in FBN1 lead to the misfolding of protein Fibrilin-1 which can't function as it is supposed to. Most of these pathogenic mutations are localized in the cbEGF (calcium-binding EGF) domains, **(Figure 1)** involved in the binding of calcium which plays an essential structural role due to its participation in disulphide bonds formation (mediated by conserved cysteine residues), intern protein interactions restriction and proteolysis protection. Then, malfunction can be explained by a reduction of the number of available Fibrilin-1, an alteration in its structure or stability of Fibrlin-1, or a problem related to its transport to the matrix by ER retention. One of the consequences of these alterations, is that due to the fact of not having enough of functional microfibrils, there is an overactivation of TGF- β, which can lead to a reduction of elasticity, overgrowth and instability of tissues (11).

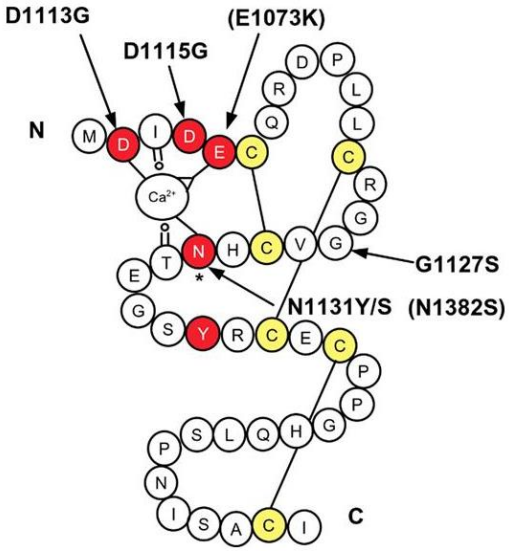

Figure 1. Representation of cbEGF13. Cisteine residues coloured in yellow (responsible for the disulphuric bonds) and N-terminal calcium-bind residues coloured in red. *(11)*

CARDIOVASCULAR MOLECULAR ALTERATIONS

MFS manifest several heart abnormalities. The major problem that MFS presents is the progressive dilation of the ascending aorta which can lead to a fatal aortic rupture or dissection. It is the most common cause of death in an adult MFS patient. Other heart problems appear in MFS; mitral valve prolapse, mitral valve calcification, Lung Artery Dilation, left ventricle hypertrophy (12)

We must consider that most of the studies realized in mice to investigate aortic dilation, aneurism and dissection, are done in the descending region of the aorta instead of the ascendant region where most of fatal aneurism take place in MFS patients. Ascending Aorta in mice is very small and this makes hard to experiment with it. This is highly important since a recent study (13) manifested differences between the regions, trying to demonstrate

differences in region and gender in murine models of MFS Fbn1$^{C1039G/+}$

Phenylephrine contractions

Regarding region differences, phenylephrine induced contractions were observed in both regions. Phenylephrine has the capacity to trigger adrenergic a-1 pathway. Interestingly, even though it is commonly known that dilation in descending aorta is the main cause that can lead to aneurism, in the ascending aorta an increase in the number of contractions takes place instead. This might cause an important weakness of the smooth muscular cells in the aortic wall. In addition, differences in the number of contractions were observed between gender, observing a larger number of contractions in male mice than in female.

COX -1 and COX-2 implication

In relation with these phenomena of contraction, in the descending aorta, seems to be related to a downregulation of COX-1 and an upregulation of COX-2. On the other hand, in the ascending aorta, there is an upregulation of COX-2 in males and a downregulation of COX-2 expression in females. This means that the expression of COX-2 can be related to the differences in the number of contractions between region and gender. Furthermore, COX-2 is correlated to a major activation of metalloproteinases, and consequently, to a major degradation and breaking down of elastic fibers.

NO levels in aortic alterations

Another factor that was considered in this study was nitric oxid (NO). High basal levels of NO were observed in MFS associated to an aortic alteration. It was concluded that high levels of NO, regulate negatively contractions induced by Phe in both sexes. In females, an upregulation of eNOS phosphorylation was noticed, probably the main cause of NO increase. Furthermore, recent studies show that a dysregulation in iNOS production plays an important role in aortic dilation in MFS.

Desmosine role in MFS

Another study focused its attention to the role that desmosine played in the aortic wall in MFS (14). Results demonstrated that the quantity of desmosine didn't differ between MFS mice and WT, although that extremely damaged elastic network was observed in MFS murine models. Another point considered in this study, was if FBN1 mutations affected the physiological cross-linking during early stages in elastogenesis or, instead, the elastic complex fragmentation in later stages. It was concluded that mutations in FBN1 didn't modify elastogenesis in early stages but it did cause an elastic complex fragmentation in later stages.

Cardiovascular pathologies

How MFS was related to certain cardiovascular pathologies such as Mitral valve prolapse (MVP), mitral valve calcification, lung artery dilation or ascendant aortic dissection and dilation, was tested in a recent study (15).

A higher prevalence of MVP was observed in MFS patients, which could be helpful to develop another diagnostic element. Calcification of the mitral valve was quite rare, so it can't be useful to identify MFS cases. A higher dilation of the lung artery was seen in MFS models and a limit was stablished so that it could be used as a characteristic to help diagnosticate MFS patients. Finally results confirmed that in MFS, the aortic wall presents a larger diameter. It is also important to mention that; MFS patients show a combination of systolic and diastolic disfunctions that are not related to a cardiac valves pathology. This can be caused by a myocardial primary contractile disfunction, resulted from an alteration of the elastic component, in

this case, probably by problems in microfibrils organization.

TGF - β Over expression

Transforming growth factor – β family is involved in the inhibition of several essential processes such as proliferation of epithelial, endothelial and hematopoietic cells, and even can stimulate the synthesis of extracellular matrix (16).

When TFG- β is secreted from the cell, it is in the context of LLC (Large Latent Complex). LLC consists of TGF- β, the TGF- β propeptide (LAP) and an LTBP. There is a smaller subcomplex called Small Latent Complex (SLC) which consists of basically LAP and TGF- β. **(*Figure 2*).**

In regard of LTBPs 1-4, they are secreted multidomain glycoproteins, three of which are directly involved in TGF- β regulation (17)

During secretion, mature TFG-β is cleaved from its propeptide although both proteins remain associated by noncovalent interactions. Then, LTBP is required for secretion and processing of latent TGF-β and it binds to LAP by disulfide bonds. At this point, signaling of TGF-β is initiated by proteolytic cleavage of LTBP, resulting in the release of latent TGF- β complex from the ECM. Continuously, TGF-β activates through LAP dissociation from the mature TGF-β Once TGF- β is released, it is able to bind to its type II receptor that leads to the phosphorylation and activation of type I TGF-β receptors. Then, this receptor can phosphorylate cytoplasmic transcriptional activation proteins such as Smad2 and Smad 3, which translocate to the nucleus and interact with gene expression (16)

In MFS, FBN1 mutations can result in a failure of matrix large latent complex of TGF-β sequestration, which increases TGF-β signaling and activation.

The precise mechanism why TGF-β is increased in MFS remains unknown. Current data is consistent with the model which correlates failure of LLC to sequestrate TGF-β, but also other mechanisms seem to be involved in excessive TGF-B activation and signaling, such as high levels of selected TGF-β activators (MMP-2, MMP-9 and thrombospondin-1) and ligands in aortic wall (18).

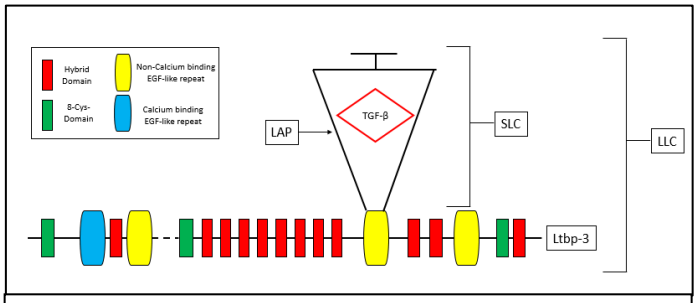

Figure 2. *Schematic representation of TGF- β's Complexes when secreted from the cell. Modified (17)*

Matrix Metalloproteinase Activity

High levels of elastolytic Matrix Metalloproteinases (MMPs) are found in the aorta of MFS patients. It appears to be related to the destruction of structural elements from the ECM. In addition, MMP-2, MMP-3 and MMp-9, are involved not only in ECM destruction but in the essential second step of the release of TGF-β; cleavage of the LAP (latency-associated peptide)

In this study (19) a non-specific MMP inhibitor was tested. It was concluded that levels of MMP-2 and MMP-9 were decreased and delayed aneurysm formation and rupture in MFS murine model.

Furthermore, we must consider that MMP-2 derives from mesenchymal cells, including Smooth Muscle Cells of the aortic media. This is relevant due to the fact that these cells are responsible for synthesis and maintenance of the complex macromolecular structure of the aorta.

Through the same testing with doxycycline (MMP non-specific inhibitor) but applied in MFS mice null for MMP-2, it was demonstrated that MMP-2 plays a specific role in TGF-β signaling and aneurysm formation, through its involvement in the activation of latent TGF-β and the increase of noncanonical signaling cascade downstream of TGF-β.

These conclusions open the possibility of developing future treatments for MFS patients based on the inhibition of MMP-2.

CURRENT PHARMACOLOGICAL TREATMENTS

Nowadays, there is no cure for MFS. That is why, the aim of pharmacological treatment is to slow or stop the development of clinical signs (above all, aortic dilation to avoid aortic dissection) of MFS (20). **(*Figure 3)***

Beta-blockers (BB)

Since probably the most relevant affectation of MFS is aortic dilation, agents which can delay or avoid it, must be considered.

Consequently, B-Blockers have always been in the first-line treatment for its capacity to reduce blood pressure by blocking the effects of epinephrine, and therefore, preventing aortic complications in MFS (21).

In summary, oral BB can reduce arterial and pulse pressures, submitting the aortic wall to less stress by decreasing the expulsion force of the left ventricle. Despite its benefits, BB therapy has recently raised doubts about its efficacy, including side-effects and intolerance that some patients experienced (20).

Angiotensin Converting Inhibitors (ACEI)

ACEI contribute to reduce arterial pressure and conduit arterial stiffness. Not only ACEI are involved in lowering the blood pressure, but presents other effects that can contribute to improve MFS conditions such as preventing cystic medial degeneration, apoptosis of VSMCs, and aortic dissection in rats, by inhibiting the activation of Ang-II AT2.

In general, there are three possible mechanisms which could explain the overall beneficial effect of ACEIs; inhibition of VSMC apoptosis, an improvement in the aortic elastic tone and the blocking of hyperhomocysteinaemia, that results in a reduction in MMP activity and a vascular stiffness increase (22).

In order to improve the efficacy of the treatment for MFS, there are occasions that they can be used in combination with BBs.

Angiotensin-II Receptor Blockers (ARBsII) - Losartan ($C_{22}H_{23}ClN_6O$)

Losartan is a selective and competitive Angiotensin type I receptor antagonist. Since Angiotensin II acts as a vasoconstrictor in vascular smooth muscle, blocking this pressor effect makes Losartan an antihypertensive treatment, promoting vasodilation and decreasing aldosterone effects (23).

Losartan can block Angiotensin II type 1 Receptor. This receptor plays an essential role in MFS pathogenesis. Considering the importance of this receptor, a study (24) was based on applying Losartan in ATR1 -/- mice and demonstrated that, firstly, loss of ATR1 fails to prevent MFS complication, and secondly, that Losartan treatment is still effective without ATR-1 target, suggesting that its therapeutic utility is off-target and ATR-1 independent.

Losartan participates in the inhibition of TGF-B expression, becoming important as a protective effect. It may be involved not only in the process of decreasing active TGF- B, but also in TGF-B's noncanonical signaling cascade

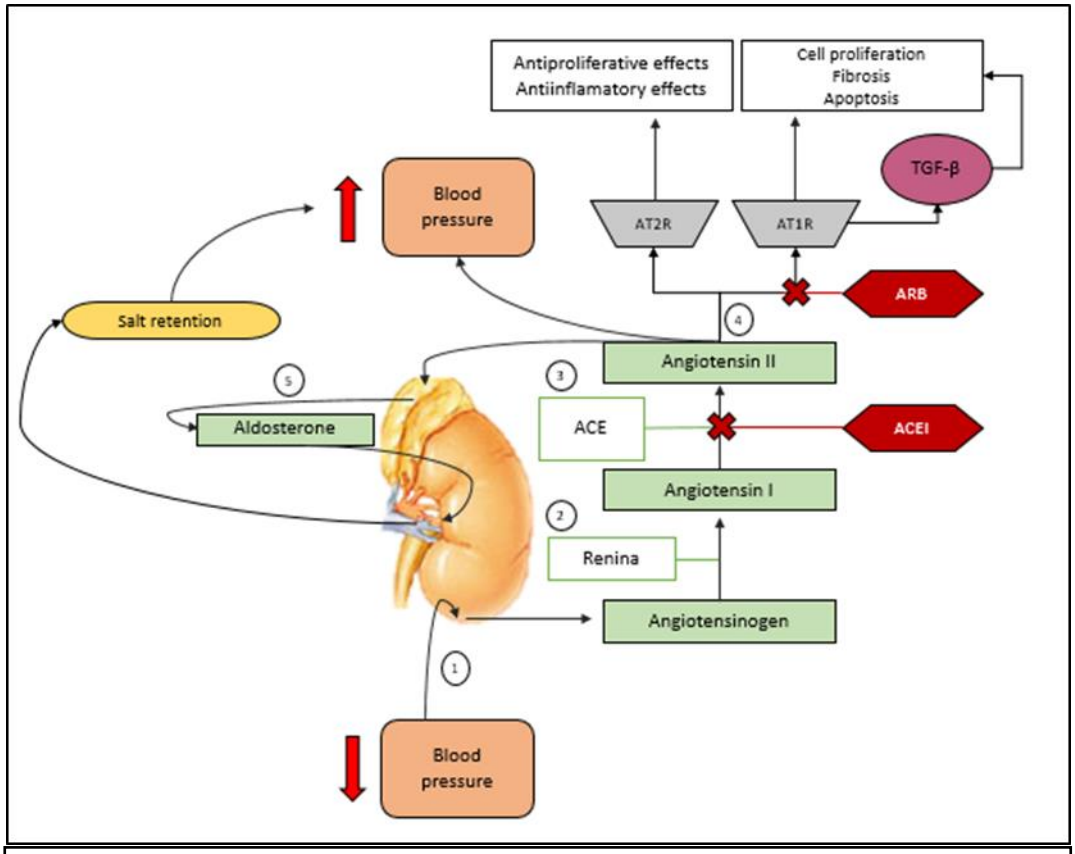

Figure 3. *Renin-Angiotensin-Aldosterone system and blocking therapeutic effect of ARBs and ACEIs. Modified (29)*

downstream (19). Interestingly, according to a recent study (25) about long term-effects of losartan on a MFS mouse model, Losartan caused a significant improvement in elastic organization, 'breaking stress' of the aortic wall was increased (meaning that it made the aortic wall structure more resistant) and decreased expression and activation of MMP-2 and 9.

It is also important to mention that Losartan was able to increase the active force of aortic smooth muscular cells at the 6th and 9th month.

On the other hand, even that endothelial dependent relaxation induced by Ach was improved by 30% for 3 months, however this improvement ends up by disappearing accompanied with an endothelial NOS reduction too.

Therefore, although it offers great advantages in a short term, when Losartan is consumed for a long period of time, beneficial effects of Losartan are significantly diminished.

TREATMENT PROPOSAL

Diagnosis and monitoring of MFS

MiR-29b

In a study with *Fbn1*$^{C1039G/+}$ mice, they found out higher levels of available TFG-β (26). This fact can lead to an NF Kappa B inhibition, due to the impossibility of phosphorylating IKKs complexes which also results in an inhibition of IK-Ba phosphorylation (Pest is blocked by NF-KB forming a dimer, kidnapping this factor), and, therefore, since NF-kB inhibits miR-29b gene expression, low levels of activated NF-kB mean high levels of miR-29b gene. This mRna is

involved in the regulation of Bcl-2 family and ECM deposition process.

ADVANTAGES	DISADVANTAGES
Liquid biopsy – Less invasive method	Unspecific – not always is MFS
Early diagnose and monitoring tool	No scientific evidence of its utility
Can prevent aortic aneurism	Need more knowledge on miRNAs
Possible future treatment	Unknown side-effects if inhibited

Table 2. *Advantages and disadvantages of miR-29b in MFS.*

It is suggested that ECM destruction, due to its participation in cellular signaling, the decrease of ECM proteins may result in Smooth Muscular Aortic Cells apoptosis. Dysregulation of these elements lead to problems in ECM aortic deposition and increases apoptosis.

Taking into account this information, a treatment based on a specific miR-29b inhibiting oligonucleotide was applied in this study and demonstrated that there is a correlation between the restriction of miR-29b and reduced levels of apoptosis.

More interestingly, it stopped the early aortic formation although it did not interfere with the late aortic formation. This might indicate that there is an aneurism formation miR-29b dependent and a non-dependent.

Although it may seem an interesting treatment approach, at the moment there is no clinical trials focusing on miRNAs for MFS (27). Surprisingly, there is another important way to apply this miRNA knowledge to MFS; early diagnosis. High levels of miR-29b may be a key indicator to diagnose and monitoring aortic formation in early stages of MFS.

Pharmacological Treatment Approach

ADVANTAGES	DISADVANTAGES
Aortic aneurysm site specific target	Does not cure MFS (MMPs inhibition)
Reduced side-effects (low dose required)	No clinical trials at present day
Scientific evidence in rats	Not tested in MFS murine models
Stopped aortic aneurysm formation	

Table 3. *Advantages and disadvantages of nanoparticles loaded with MMP inhibitors conjugated with elastin ab treatment in MFS.*

As stated before, MMPs are found in a higher level in MFS model mice than in wild-type, and that leads to destruction of elements involved in ECM. Therefore, a treatment directed to inhibit MMPs could be an attractive approach to mitigate aortic wall damage. Furthermore, there are two specific MMPs (-2 and – 9) that, as mentioned before, are also involved in TGF-B signaling and activation, and inhibiting them has been shown that offers significant beneficial effects in aortic complications in MFS.

In recent studies, the effect of systemic treatments of MMP inhibitors have shown promising results as it decreased and delayed aortic aneurysm formation in MFS murine model. The problem appears when trying to translate this results to clinical practice; considerable side-effects due to systemic affectation or, on the other hand, doses used were too low.

That is why, a directed therapy based on targeted nanoparticles (NPs) loaded with low doses of MMP Inhibitor was proposed. This way, side-effects and doses related problems would be solved by targeting directly to the site of an

aneurysm in the abdominal aorta through elastin antibodies.

In this study (28), intravenous injections of nanoparticles loaded with Batimastat (MMP Inhibitor) conjugated with elastin antibodies, were delivered in rats. Results showed that the targeted therapy achieved activity MMP inhibition, stopped elastin degradation, calcification and aortic aneurysmal formation at a low dose of BB-94.

To confirm the beneficial effect of NPs therapy in relation to systemic administration, same dose administered before in NPs, was delivered systemically and it was concluded that it was ineffective to inhibit MMPs.

We must consider the fact that these conclusions were obtained from non-MFS mice rats.

So, knowing that the aim of MFS treatment is to delay or stop aortic aneurysm formation and aortic wall degradation, applying nanoparticles loaded with MMP inhibitors (specially MMP-2 and -9) and conjugated with elastin antibodies to target the aneurysm site, in MFS patients appears to be an interesting treatment approach.

CONCLUSIONS

We have just started to deeply understand the mechanisms involved in MFS. Not many years ago, MFS used to be diagnosed by phenotypical characteristics, which, nowadays, has radically been substituted by genetic evidence; FBN1 mutations.

As already mentioned before, MFS can be caused by more than a thousand point mutations in the FBN1 gene that lead to a misfunction of the Fibrilin-1 protein. Therefore, it must be taken into consideration that there is more than one mutation that can cause MFS and this fact is a problem when trying to do research on this syndrome and obtaining specific results of each case since representing all possible mutations is not viable (studies are usually based on murine models with one specific knockdown).

That is why, describing MFS phenotype has not been a problem since it is already well-stablished but where more differences seem to appear and most discussion is centered, is concerning the molecular mechanisms involved in this syndrome. While some affected elements are already widely known, such as microfibers anomalies and TGF-β upregulation (although the exact pathogenic mechanism still remains unknown), recent studies suggest that there are many other relevant elements dysregulated in this syndrome; NO, MMPs, COX-2, … Most studies focus on the most severe consequence of MFS; aortic aneurysm.

That is the main reason why treatments are mainly focused on preventing that condition. Since the understanding of MFS basis has just started, pharmacological approaches are still mainly palliative (Losartán, Verapamil, Atenolol, …), which do not solve original MFS disorders.

That explains why, for now, treatment of MFS should be based on individual features and characteristics to solve symptoms in a personalized way. Always being aware of aortic wall changes (dilation) to administrate BB, ARB (II) to prevent further complications. Furthermore, when excessive aortic dilation takes place, the Gold Standard technique remains as the aortic prothesis implementation.

So, therefore, close monitorization of patient's evolution is key to prevent further aortic complications. A possible way to obtain information of the development of the syndrome in an easy, economical and painless method, is by detecting specific miRNAs (over or under expressed in MFS; **MiR-29b** by liquid biopsy which will give us

information about the early formation of an aortic aneurysm.

Regarding new pharmacological strategies, they should be based on the most recent publications since knowledge in MFS is increasing year per year. Yet, considering that there are molecular mechanisms that are involved in MFS but are not yet completely understood, elaborating a pharmacological solution which depends on unknown molecular factors, is a major obstacle that must be solved.

Even though all the aforementioned drawbacks when trying to suggest a new pharmacological treatment, inhibiting MMPs can be an attractive alternative treatment strategy. To avoid causing excessive side-effects and being able to obtain better therapeutic response with less dose, treatment based on NPs loaded with Batimastat and conjugated with elastin antibodies, could be an effective and interesting approach to improve quality and life expectancy in MFS patients. Of course, more research using MFS murine models needs to be done.

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