HYPERTROPHIC CARDIOMYOPATHY

THE CHANGES IN THE NON-SARCOMERIC CYTOSKELETON LINKED TO THE NUCLEUS

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Master thesis

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Table of content

Summary

Hypertrophic cardiomyopathy (HCM) is an inheritable cardiovascular disease which is characterized by thickening of the septum and left ventricular wall, leading to impaired relaxation and obstruction of the left ventricular outflow tract. Recent research shows that alterations in the non-sarcomeric cytoskeleton, including microtubules and intermediate filaments, play a role in the pathogenesis of HCM by their significant increase in HCM and thereby contributing to the observed nuclear deformations.

This study aimed to investigate the changes in the non-sarcomeric cytoskeleton in HCM cardiomyocytes and how they are related to the deformations of the nucleus. Three main objectives were included: First, separating the polymerized and free form of tubulin from each other to examine polymerized tubulin protein levels and correlate them with nuclear parameters . Secondly, examining nuclear proteins lamin A/C and γH2AX levels and their correlation with nuclear parameters and non-sarcomeric component levels. Third, assessing the effect of different compounds affecting microtubules on the contractility of cells using a mouse model.

Although the results of the first objective were inconclusive due to the issue that the polymerized and free fraction of tubulin could not properly be separated, the study found an increase in nuclear proteins lamin A/C and γH2AX levels in HCM compared to non-failing (NF) donors. Furthermore, the correlations suggest that lamin A/C acts as a protective mechanism for chromatin against mechanical stress applied on the nucleus to prevent DNA damage. The mouse model suggests that stable microtubules support cardiomyocyte contractility, and that detyrosination contributes to impaired contraction in cardiomyocytes, but further testing is needed to draw more reliable conclusions.

Together, the study aims to shed more light on the molecular mechanisms underlying HCM, specifically focusing on the interplay between the cytoskeleton and the nucleus. Overall, the goal was to contribute to the identification of potential therapeutic targets for regenerative treatments, and in the end improve patient outcomes.

Samenvatting

Hypertrofe cardiomyopathie (HCM) is een erfelijke cardiovasculaire aandoening die wordt gekenmerkt door verdikking van het septum en linker ventrikelwand, wat leidt tot verminderde relaxatie en obstructie van de linker ventrikel uitgang. Recent onderzoek heeft aangetoond dat veranderingen in het niet-sarcomere cytoskelet, dat onder meer microtubuli en intermediaire filamenten bevat, een rol spelen in het ziekteverloop van HCM doordat ze significant zijn toegenomen en daardoor bijdragen aan de waargenomen nucleaire vervormingen in de cardiomyocyt.

Het doel van dit onderzoek was om de veranderingen in het non-sarcomerische cytoskelet in HCM cardiomycyten verder te onderzoeken en vervolgens te analyseren hoe deze veranderingen gerelateerd zijn aan de vervormingen van de celkern. Er waren 3 hoofddoelstellingen: Ten eerste, het scheiden van de gepolymeriseerde en vrije vorm van tubuline om gepolymeriseerde tubuline eiwit niveaus te onderzoeken en deze te correleren met nucleaire parameters. Ten tweede, het onderzoeken van de niveaus van nucleaire eiwitten lamin A/C en γH2AX in HCM cardiomyocyten en hun correlatie met nucleaire parameters en niveaus van niet-sarcomere eiwitten. Ten derde, het analyseren van het effect van verschillende behandelingen die microtubuli als doelwit hebben, op de contractiliteit van geïsoleerde wild-type muis cardiomyocyten.

Hoewel de resultaten van de eerste doelstelling niet betrouwbaar waren, omdat de gepolymeriseerde en vrije vorm van tubuline niet goed konden worden gescheiden, toonden de kern eiwit analyses een

toename van nucleaire eiwitten lamin A/C en yH2AX niveaus aan in HCM in vergelijking met niet falende donoren. Bovendien suggereren de correlaties dat lamin A/C fungeert als een beschermingsmechanisme voor chromatine tegen de krachten uitgeoefend op de kern om DNA-schade te voorkomen. Het muismodel suggereert dat stabiele microtubuli bijdragen aan een betere contractie van de cardiomyocyt en dat detyrosinatie vermindering van contractie veroorzaakt, maar meer uitgebreid onderzoek is nodig om betrouwbaardere conclusies te trekken.

Al met al beoogt de studie de moleculaire mechanismen die ten grondslag aan HCM liggen in kaart te brengen, waarbij de aandacht specifiek uitgaat naar de wisselwerking tussen het cytoskelet en de celkern. Het algemene doel was bij te dragen aan het vinden van potentiele therapeutische doelwitten in de cel voor regeneratieve medicatie en behandeling en uiteindelijk de prognose van HCM patiënten te verbeteren.

Background

Hypertrophic cardiomyopathy (HCM) is the most prevalent inherited cardiac disease, with an incidence of 1 in 500 people (1). HCM causes the heart muscle to thicken, especially in the left ventricular wall and septal wall as can be seen in figure 1. The increased thickness can lead to obstruction of the left ventricular outflow tract and impairs the relaxation of the left ventricle. The result is that the heart must work harder to pump enough blood (2). Shortness of breath, chest pain, or alterations in the heart's electrical system are all signs of HCM. HCM can lead to arrhythmias, heart failure or even abrupt death (2). In ~50% of the cases of HCM, the cause of the disease is a sarcomere gene mutation, while in the other ~50% there is no known mutation (3). The mutation is mostly found in the beta myosin heavy chain (MYH7) or cardiac myosin-binding protein-C (MYBPC3) gene. Other known mutations are cardiac troponin T (TNNT2) and α-cardiac actin (ACTC1) gene mutations (2-4).

To reduce the symptoms of HCM, medications such as beta blockers, calcium channel blockers or heart rhythm drugs are readily available (5, 6). When medication is unable to significantly reduce outflow tract obstruction, a septal myectomy, an open-heart procedure, may be performed. During the surgery a part of the thickened septum will be removed to improve the blood flow through the heart (5, 6).

When HCM occurs, the cause can be found at the level of the cardiomyocytes. Cardiomyocytes are the contracting cells and the building blocks of cardiac muscle (7). Figure 1 shows the phenotype of the cardiomyocytes in non-failing heart tissue compared to the hypertrophic cardiomyocytes in HCM heart tissue.

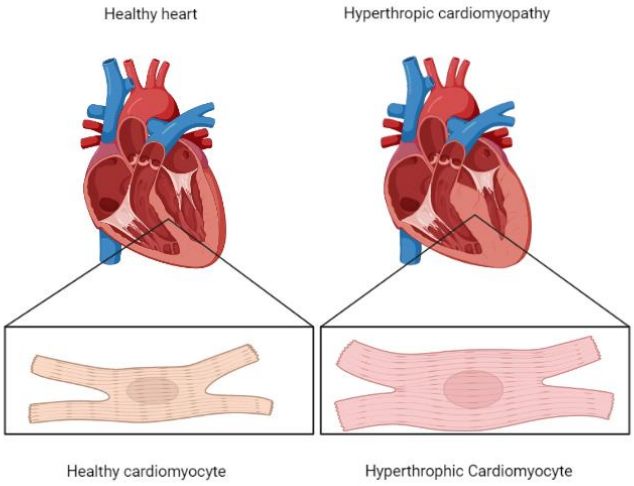


Figure 1: healthy heart vs HCM heart, the septum is thicker. The HCM cells are also bigger compared to the nonfailing cells.[Biorender®, j.s.bloem]

Cytoskeletal proteins in cardiomyocytes

The contraction of cardiomyocytes is powered by the cytoskeleton, which can be divided into two groups. First, the contractile cytoskeleton which consists of repeated arrays of actin and myosin that form the basic contractile units of striated muscle, these units are known as sarcomeres. Second, there is the non-sarcomeric cytoskeleton, which consists of actin filaments (microfilaments), microtubules (MTs) and intermediate filaments (IFs). The non-sarcomeric cytoskeleton provides mechanical support, plays an important role in tension-sensing and signal transduction, provides a transport network for proteins and organelles, and they contribute in cell division and mobility as well (7, 8) .

Microtubules

A large part of the cytoskeleton consists of MTs. MTs are present in all eukaryotic cells and play a role in mitosis, cell motility, intracellular transport, and cell shape preservation. As shown in figure 2, dimers of α- and β-tubulin form the building blocks of MTs, which exist in its free form or in its polymerized form to become MTs. α - and β -tubulin subunits are organized into linear protofilaments to become MTs. Each MT is made up of 10 to 15 protofilaments. MTs are structures that can quickly expand through polymerization or shrink by depolymerization (figure 2). A MT has a plus and a minus end, the plus end grows faster than the minus end and the minus end is often anchored to a structure called the MT organizing center (MTOC). MTs often undergo post translational modifications (PTMs), which increase the functional variety of MTs. In addition, PTMs have an impact on the organization, dynamics, and interactions of the MT with other cellular elements (7). The PTMs focused on in this study are detyrosination and acetylation. Detyrosination stabilizes microtubules by preventing them to disassemble and can connect microtubules to the Z-discs of the sarcomere through desmin (an IF) (9). As a result, the stability and stiffness of the cardiomyocytes are increased. Acetylation increases microtubule flexibility which makes microtubules more resistant to mechanical stress and possible damage due to mechanical stress (10). In HCM MTs and its PTMs are increased, the enhanced MTs and its PTMs detyrosination and acetylation of α-tubulin contribute to the malfunction of the cardiomyocyte in HCM (7).

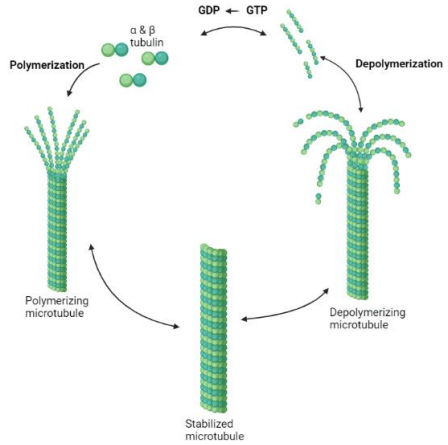


Figure 2: structure of microtubules, they consist of α and β tubulin, which can be altered by PTMs. The tubulins can grow by polymerization and shrink by depolymerization. [biorender®, j.s.bloem]

Intermediate filaments

Intermediate filaments contribute to the mechanical stability of the cell. The interaction of IFs with each other results in maintenance of cellular structure and shape and also influences cellular signaling and gene expression due to mechanotransduction. The IFs in the non-sarcomeric cytoskeleton of cardiomyocytes are mostly desmin (11, 12). Moreover, desmin is linked to MTs, and together they play a key role in maintaining the structural integrity and function of cardiomyocytes. In HCM their levels are elevated, which can be destructive to the heart. Disturbance in the cell's mechanical forces due to the altered levels of IFs and MTs can result in increased forces being applied on the nucleus, which can alter the nuclear structure and genome organization (11-13).

Actin filaments

Lastly, actin filaments are the smallest filaments in the non-sarcomeric cytoskeleton, they are also part of the sarcomeric cytoskeleton where they form together with myosin, the sarcomere (the contractile units in the cell). The non-sarcomeric actin filaments contribute to cell shape and structure, movement and division and intracellular transport (14). Actin binding proteins can affect how the filaments are arranged. Mutations in the ACTC1 gene lead to alterations in interactions of actin with myosin, and plays a role in some variants of HCM (4).

Nuclear mechanotransduction

Nuclear mechanotransduction refers to the process by which mechanical forces are transmitted across the nuclear envelope and into the nucleus, where they can affect gene expression and other nuclear processes. This process is important for a variety of biological processes, including cell migration, tissue development, and mechanosensing (15). In HCM there are alterations in the mechanical stress within the cardiomyocyte due to increases of sarcomeric components, this may affect mechanotransduction and therefore cell signalling pathways, resulting in altered gene expression patterns.

The transduction of mechanic signals across the cytoskeleton to the nucleoskeleton is done by the linker of nucleoskeleton and cytoskeleton (LINC) (12, 16). This so called LINC complex is located in the nuclear envelope, which separates the cytoplasm from the nucleus. The function of the LINC complex is to give shape and structure to the nucleus, and to connect the previous mentioned non-sarcomeric components to the nuclear lamina, a network of intermediate filaments that supports the nuclear envelope and are connected with intranuclear structures like heterochromatin. Figure 3 shows the LINC complex; the nuclear lamina consists of Lamin A/C and is located underneath the inner nuclear membrane. SUN1/2 proteins connect the nuclear lamina to the inner nuclear membrane. In turn, the SUN proteins bind to the outer nuclear membrane via KASH to nesprin. Nesprin can bind to the MTs, IFs (in this figure desmin) and actin that together make up the non-sarcomeric cytoskeleton (16). Recent developments have given intriguing new insights into how the LINC complex functions to mechanically regulate the numerous roles of DNA in cells from mechanically activated tissues including skeletal and cardiac muscle (12, 16). However, there are still some gaps in our knowledge of these mechanisms, studying the LINC complex might help to obtain more knowledge about the pathogenesis of cardiac diseases like HCM.

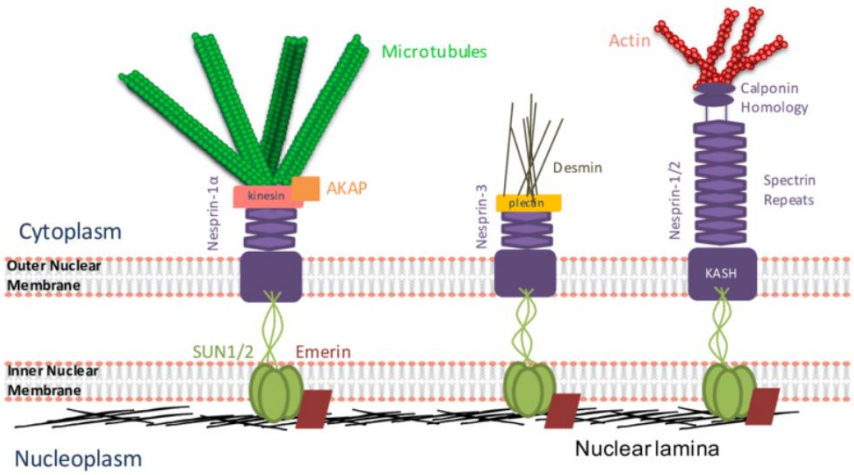
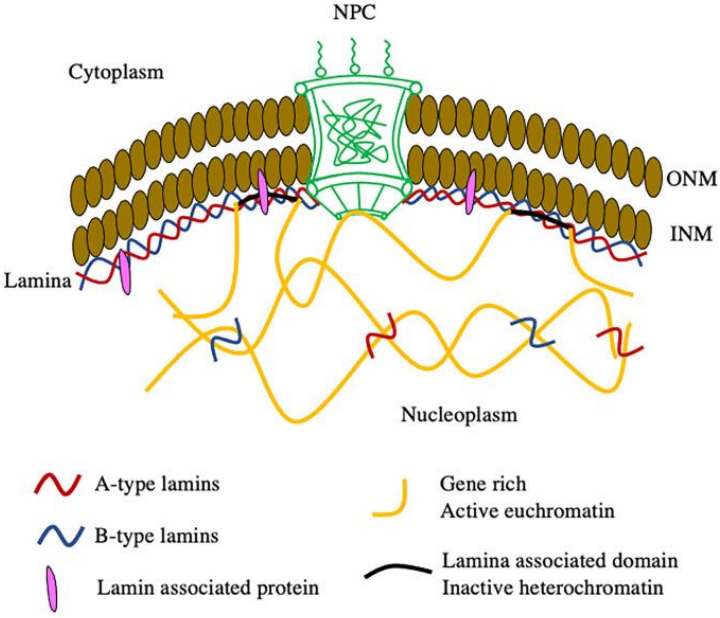


Figure 3: The LINC complex, the nuclear lamina binds at the inner nuclear membrane via SUN1/2 proteins, which in turn bind to the outer nuclear membrane via KASH to nesprin. Nesprin can in turn bind to the cytoskeleton components of the cell; MTs, IF desmin and actin (16).

Nuclear proteins in cardiomyocytes

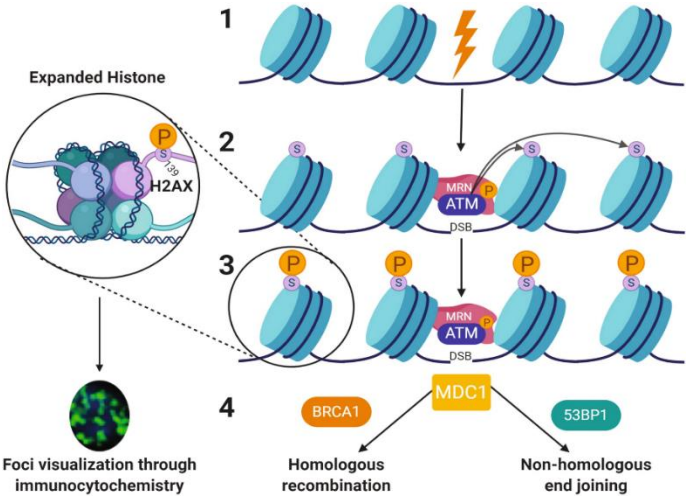
The non-sarcomeric components are via the LINC complex connected to nuclear proteins such as lamin A/C. Furthermore, increased forces applied by the non-sarcomeric components might induce DNA damage and the activation of its repair mechanisms, which can be characterized by the DNA damage protein γH2AX.

Lamin A/C is an IF that plays an important role in the structural organization of the nucleus in cells and is located underneath the nuclear membrane, see figure 4. Lamin A/C is encoded by the LMNA gene and is expressed in a wide range of cell types. Mutations in the LMNA gene have been linked to a variety of disorders, including hypertrophic cardiomyopathy in some cases. Studies have shown that abnormalities in Lamin A/C can disrupt the normal function of cardiomyocytes (17, 18). This disruption can lead to changes in the mechanical properties of the cells, as well as alterations in cell signaling pathways and gene expression. While it is known that Lamin A/C is connected to the non-sarcomeric components of the cytoskeleton through the LINC complex, there is little understanding of the role of this interaction in the development of HCM.



Figuur 4: Lamin A/C organization in the nucleus (19).

Lamin A/C is connected to heterochromatin. The forces lamin A/C is exposed to can lead to DNA damage, which can be detected by γH2AX, a marker of double-strand DNA breaks. As can be seen in figure 5 γH2AX is a phosphorylated form of histone H2AX that is recruited to DNA damage sites, where it helps to initiate the DNA damage response. It is typically used as a marker of DNA damage and repair processes in various types of cells, including cardiomyocytes. γH2AX has been used as a tool for studying the role of DNA damage response pathways in cardiomyocyte function and survival (20, 21). The level of γH2AX is increased in HCM (22). This increase may be a consequence of increased DNA damage in HCM hearts, possibly as a result of increased stress and stiffness in the cells or impaired DNA repair mechanisms. This suggests that DNA damage and repair mechanisms may be involved in the pathogenesis of HCM.



Figuur 5: H2AX phosphorylation after double stranded DNA break resulting in γH2AX, a marker for double stranded DNA breaks which is headed to the site of DNA damage and puts DNA repair mechanisms into operation. (23)

Problem & Goal

The complete understanding of the molecular mechanisms involved in HCM still remains unclear. Studying the components present in the cytoplasm of the cardiomyocytes, such as microtubules, intermediate filaments, and actin filaments, may offer useful insights into these mechanisms. Additionally, investigating the interplay between cytoplasmic and nuclear proteins in healthy and diseased cardiomyocytes may provide further understanding of the molecular pathogenesis of HCM and thereby possibly identifying new targets for therapeutic intervention.

Recent studies have demonstrated that there is a dramatic alteration in the cardiomyocyte cytoskeletal landscape in human heart failure and also specifically in hypertrophic cardiomyopathy (HCM). There is a significant increase in the expression of MTs and IFs, while there is also an increase in detyrosination and acetylation on MTs. This is accompanied by a loss of sarcomere (3, 12, 24). As a result hypertrophic cardiomyocytes become more stiff and contraction and relaxation is slowed down.

The role that the interaction of the nucleus and its nuclear proteins with the non-sarcomeric cytoskeleton plays in the development of HCM is still not fully mapped out. Recent studies have shown evidence for nuclear abnormalities in HCM, and how this has to do with the changes in MT expression. Figure 6 illustrates that the nuclei in HCM cardiomyocytes show deformations, in other words invaginations. These results are the first step towards linking the nucleus to HCM and finding novel therapeutic targets. The next step is to get a better understanding of the interaction between non-sarcomeric cytoskeletal changes and the nucleus in HCM.

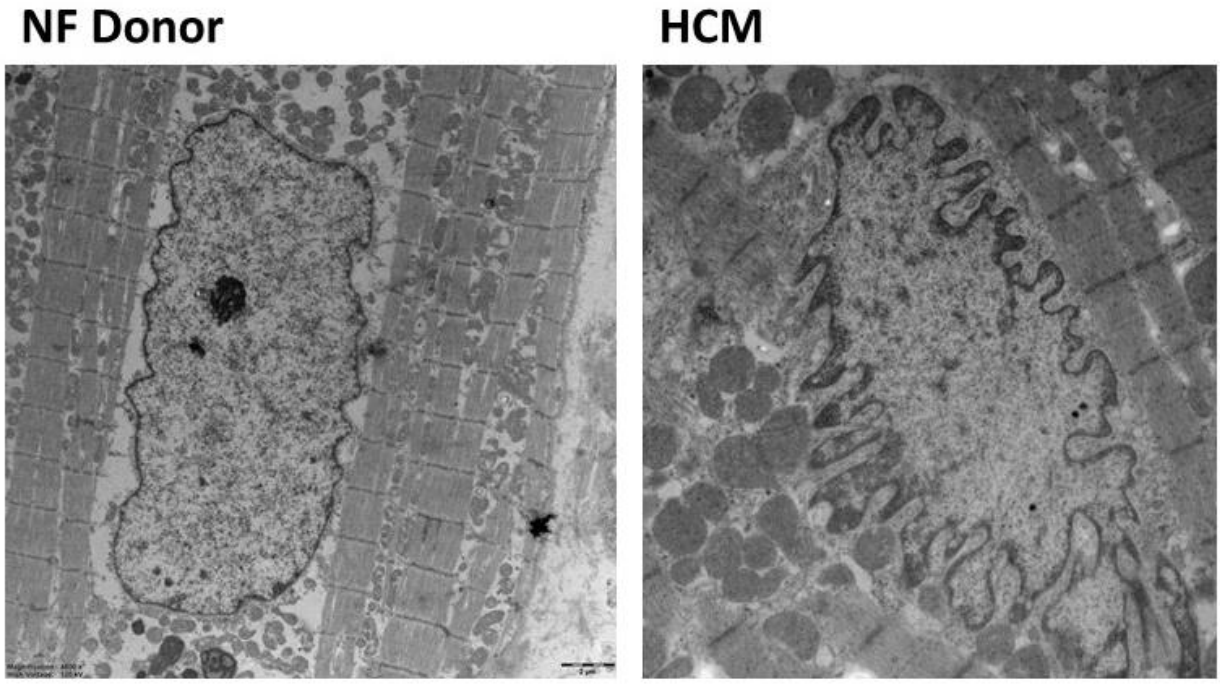


Figure 6: EM images of cardiomyocyte nuclei from both NF donor and HCM cells. HCM nuclei are deformed and show invaginations, while NF donor nuclei show a more smooth membrane [physiology department results].

The main focus of this study was to investigate how the changes in the non-sarcomeric cytoskeleton in HCM cardiomyocytes are related to the deformations of the nucleus. To address this research question, the study has outlined the following sub-questions:

1. Which fraction of total tubulin concentration is in its polymerized form, and how are these polymerized tubulin protein levels correlated to the deformations of the nucleus?
2. How are other nuclear proteins expressed in HCM cardiomyocytes and how does this correlate with alterations in the nuclear structure and cytoskeletal components?
3. What impact do different compounds that manipulate microtubules have on the functional properties of cardiomyocytes?

In subquestion 1 we were particularly interested in the polymerized form of tubulin, as it plays a critical role in the stiffening of cells and potentially contributes to the nuclear deformations in HCM. Subquestion 2 should tell us if there are any possible links between alterations in nuclear protein levels and changes in nuclear structure and non-sarcomeric components. The found correlations studied in subquestion 1 and 2 can shed light on the molecular mechanisms of HCM and contribute to find potential therapeutic targets. Subquestion 3 should tell us the possibility of enhancing the functioning of cardiomyocytes by manipulating MTs and determining the most effective approach for doing so.

Methodical approach

To investigate the previous mentioned research question and sub-questions, cardiomyocytes were subjected to functional analysis (contractility) and protein analysis (Western Blot). Human patient material and a mouse model were used for testing.

In detail this included the analysis of microtubule-, Lamin A/C and DNA damage protein γH2AX levels in nonfailing donor myocardium and HCM septal heart tissue to investigate its role in the nuclear abnormalities observed. To carry out the various analyses, western blot was used to study protein levels in nonfailing donor cardiomyoctes and HCM septal heart tissue. The protein levels were correlated to previous obtained nuclear parameter data to investigate possible links.

Furthermore, an animal model was used in which wild type (WT) mice hearts were isolated and the cardiomyocytes were subsequently exposed to compounds that manipulate MTs to study its effect as therapeutic intervention. Unloaded shortening measurements were performed to test the effect of these interventions on contraction and relaxation of cardiomyocytes.

Materials and method

Human tissue analysis

Human tissue was used which was obtained during myectomy surgery. The samples of human cardiac tissue were collected from the interventricular septum (25). Table 1 lists the patient characteristics. This study analyzed a total of 19 samples obtained from individuals with HCM and 8 samples from non-failing (NF) donors with no known history of cardiac abnormalities obtained from the University of Pennsylvenia , which served as controls. Among the HCM patients, there were 10 individuals who carried a mutation and were therefore sarcomere mutation positive (SMP), and 9 individuals where no mutation was found and were therefore sarcomere mutation negative (SMN) patients. The SMP group included individuals carrying various mutations, including 1 MYL2 mutation, 1 TNNT2 mutation, 1 MYH6 mutation, 2 of the MYH7 mutation, and 5 of the MYBPC3 mutation. The HCM samples were compared to the NF donor samples.

Table 1. Patient characteristics (n=27)

Characteristics	**Total HCM**	**HCM_{SMP}**	**HCM_{SMN}**	**NF donor**
Total patients	19	10	9	8
Males (%)	13 (68%)	7 (70%)	6 (66.6%)	-
Females (%)	6 (32%)	3 (30%)	3 (33.3%)	-
Mean age at operation (+-SD, range)	55.9 (+-18.4, 15-75)	48.7 (+-19.7, 15-72)	63.9 (+-13.8, 37-75)	-
Mutation				
MYL2	-	1	-	-
TNNT2	-	1	-	-
MYH6	-	1	-	-
MYH7	-	2	-	-
MYBPC3	-	5	-	-
IVS (+-SD)	20.8 mm (+-5.7)	22.8 mm (+-6.8)	18.7 mm (+-3.2)	-
IVS/BSA	10.8 (+-3.42)	11.6 (+-4.4)	9.9 (+-1.6)	
Pathogenicity *(n)*				
Class 3 (unknown significance)	-	2	-	-
Class 4 (likely pathogenic)		1		
Class 4/5 (likely/pathogenic)	-	4	-	-
Class 5 (pathogenic)	-	3	-	-

Sample preparation

Regular sample preparation (26)

Frozen tissue was lysed in 40 µL lysis buffer (25% NuPage 4x, 10% (w/v) DTT 10x, 65% MilliQ)/mg tissue. Tissue was homogenized with a grinder and transferred to 1.5 mL microcentrifuge tubes per sample. Samples were heated for protein denaturation at 99 °C for 5 minutes and centrifuged at 25 °C for 10 minutes (15000 rpm). Supernatant was collected and stored at -80C.

Free and polymerized tubulin separation_ tissue preparation

Figure 7 shows the sample preparation protocol for the separation of free and polymerized tubulin. Frozen tissue was lysed in 50 µLlysis buffer MTSB(microtubule stabilizing buffer) /mg tissue, supplemented with protease inhibitor and GTP, a control without GTP was included. Tissue was homogenized with grinder and transferred to 1.5 mL microcentirifuge tubes per sample. Soluble free tubulin and insoluble polymerized tubulin were separated by centrifugation at 25 °C for 15 min (15.000 rpm). Supernatant was collected and identified as free tubulin fraction sample. 50 µL1x RIPA + 0.1%SDS (Cayman Chemicals)/mg tissue, was added to the remaining pellet. Sample was vortexed until the pellet was maximally solubilized (takes appr. 30 min – 1hour.). Debris was removed by centrifugation at 4 °C for 15 min (15.000 rpm). Supernatant was collected and identified as polymerized tubulin fraction sample. All samples were stored at -80 °C.

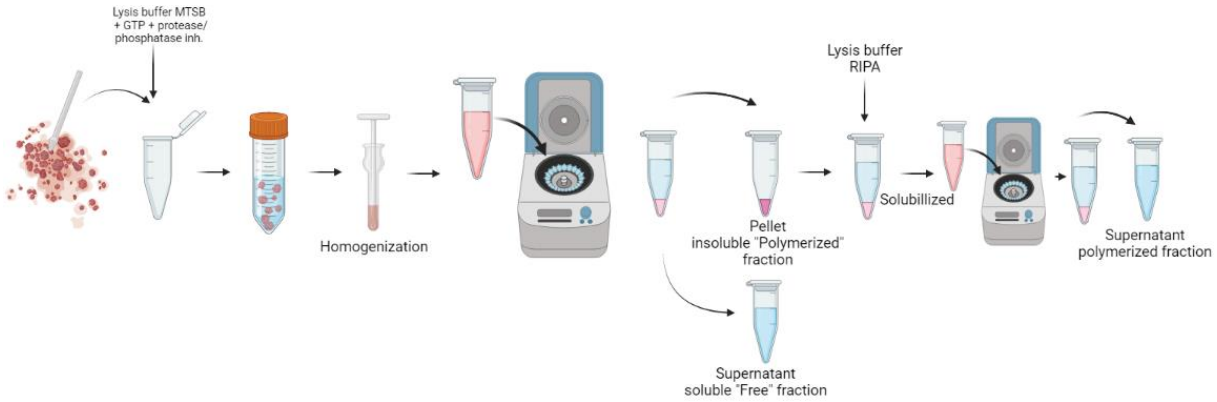


Figure 7: Sample preparation protocol for separation polymerized and free fraction of tubulin [biorender®, j.s.bloem]

Western blot

A general western blot protocol was performed (27). Samples were diluted in RSB (Reducing sample buffer) dilution liquid (25% NuPage 4x, 10%DTT 10x, 65%MilliQ) and loaded on 8-16% or 4-15% gels along with a molecular weight marker (Biorad Precision plus protein dual color standards #161-0374). Samples were loaded and transferred onto PVDF membranes. After blocking in 5% milk- or 3% bovine serum albumin (BSA) in 1x TBS-T (20% 1M Tris-HCL (pH 7.5-7.6), 8% NaCl, 0.1% tween) , membranes were first incubated with primary antibodies overnight, followed by secondary anti-rabbit or anti-mouse antibodies for 1h. The used antibodies are given in table 2. Visualization was done with an enhanced chemiluminescence detection kit (Amersham), scanned with Amersham Imager 600 (GE healthcare) and quantified by analysis software for gel and blot images ImageQuant (ImageQuant TL, GE Healthcare). To account for variations in loading, protein quantities were normalized to GAPDH or α-actinin.

Table 2. List of antibodies used

	Primary	**Secondary**
α-tubulin	α-tubulin sigma aldrich T9026 dilution: 1:5000	Polyclonal goat Anti-mouse agilent dako P0447 dilution: 1:2000
Detyrosinated α-tubulin	Detyrosinated α-tubulin Abcam AB48389 dilution: 1:1000	Polyclonal goat Anti-Rabbit agilent dako P0448 dilution: 1:2000
Acetylated α-tubulin	Acetylated α-tubulin sigma Aldrich T7451 dilution: 1:2000	Polyclonal goat Anti-Rabbit agilent dako P0448 dilution: 1:5000

Lamin A/C	Lamin A + Lamin C Abcam ab108595 Dilution: 1:5000	Polyclonal goat Anti-Rabbit agilent dako P0448 dilution: 1:2000
γH2AX	γH2AX Sigma Aldrich S139P Dilution: 1:1000	Polyclonal goat Anti-mouse agilent dako P0447 dilution: 1:2000
GAPDH	GAPDH cell signaling 2118s Dilution: 1:5000	Polyclonal goat Anti-Rabbit agilent dako P0448 dilution: 1:5000
α-actinin	Alpha-Actinin cell signaling 3134S Dilution: 1:1000	Polyclonal goat Anti-Rabbit agilent dako P0448 1:2000

Correlation analysis

Pearson correlation was used to see if the measured protein levels in both HCM and NF donor correlated with the parameters of the nucleus and the non-sarcomeric components of the cardiomyocyte. The correlation coefficient (r) is a value that can range from -1 to +1, where values closer to 0 indicate little to no correlation between the variables. A correlation with an absolute value greater than 0.5 is generally considered to indicate a relationship between the two variables being examined. Table 3 and 4 contain previously obtained nuclear data and non-sarcomeric component data respectively. The nuclear parameters assessed in this study included the number of invaginations in the nucleus per µm, the percentage of heterochromatin present in the border area of the nucleus (100 µm thick), the total percentage of heterochromatin in the entire nuclear area and the roughness of chromatin. The characteristics of the nuclear parameters are given in table 3 and clarified in figure 8. Additionally, the measured protein levels were examined for correlations with the intraventricular septum thickness (IVS), as presented in table 1 patient characteristics.

Furthermore, the non-sarcomeric components analyzed for correlations were α-tubulin, detyrosinated α-tubulin, acetylated α-tubulin, and desmin. The levels of these proteins were previously obtained by western blot results and are presented normalized to the protein levels in NF donors. The characteristics of the levels of the non-sarcomeric components are given in table 4.

Table 3. Nuclear characteristics.

Nuclear characteristics	**Total HCM**	**HCM_{SMP}**	**HCM_{SMN}**	**NF donor**
Invaginations per µm (#)	0.32 (+-0.09)	0.32 (+-0.11)	0.32 (+-0.06)	0.18 (+-0.03)
Percentage area heterochromatin in border area (%)	71.73 (+-5.13)	71.8 (+-4.23)	71.65 (+-6.25)	76.34 (+-10.21)
Percentage area heterochromatin in nucleus (%)	20.98 (+-4.26)	20.56 (+-5.1)	21.45 (+-3.34)	24.39 (+-10.45)
Roughness chromatin (a.u.)	5.15 (+-1.39)	5.28 (+-1.60)	5.02 (+-1.19)	3.56 (+-1.25)

Table 4. Levels of non sarcomeric compontents normalized to NF donor.

Non sarcomeric components normalized to NF donor	Total HCM	HCM_{SMP}	HCM_{SMN}	NF donor
α-tubulin	2.83 (+-1.28)	3.16 (+-1.51)	2.46 (+-0.9)	1 (+-0.57)
Detyrosinated tubulin	5.76 (+-4.69)	8.07 (+-5.41)	3.2 (+-1.57)	1 (+-0.75)
Acetylated tubulin	42.84 (+-28.39)	52.82 (+-29.5)	31.74 (+-23.92)	1 (+-0.52)
Desmin	1.42 (+-0.37)	1.54 (+-0.39)	1.29 (+-0.32)	1 (+-0.47)

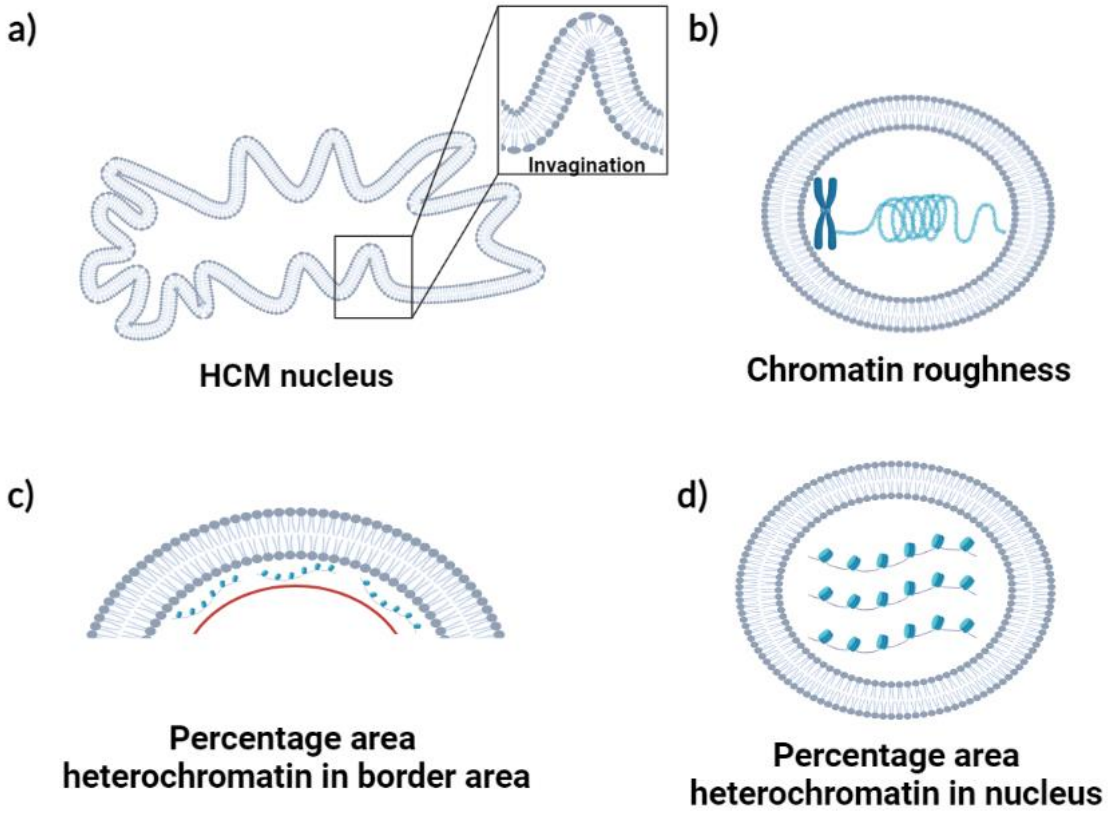


Figure 8: Schematic image of nuclear parameters which alter in HCM cardiomyocytes. These nuclear parameters are examined for correlations with protein levels in HCM cardiomyocytes and NF donor cardiomyocytes. The following are shown: a) Invaginations or deformations present in the HCM nucleus. b) The density of chromatin, which refers to how tightly packed chromatin is within the nucleus. c) The percentage area heterochromatin in the border area, which indicates how much of the border area is covered with heterochromatin. The border area is indicated by the area between the red line and the membrane. d) The percentage area heterochromatin in the entire nucleus, which indicates how much of the whole nucleus is covered with heterochromatin. [biorender®, j.s.bloem]

Mouse tissue analysis

C57BL/6J wild type (WT) mice hearts were isolated using Langendorff perfusion, which is a laboratory technique used to perfuse an isolated heart with a nutrient-rich buffer solution in order to keep it alive so cardiomyocytes can be studied (28, 29).

Cytocypher

As can be seen in figure 9, 11 WT mice cardiomyocytes were isolated and plated on single well dishes. After 1h. the cardiomyocyte plating medium was replaced with either control medium or medium containing one of three compounds (nocodazole, epo-y, and taxol) whose effects were being studied. Nocodazole is known to destabilize MTs and depolymerizes them, epo-y is known to block detyrosination of tubulin, and taxol is known to stabilize MTs (30, 31). Nocodazole or epo-y treatment was given to the cardiomyocytes of 6 of the mice, while taxol treatment or a simultaneous treatment of taxol and epo-y was given to the cardiomyocytes of the other 5 mice. The compound was incubated for 1 hour for nocodazole and taxol, and for 2 hours for epo-y and taxol + epo-y. After incubation with the compound, the medium was switched to tyrode which contained calcium for contraction. The dishes were loaded one at a time in the cytocypher system. The cardiomyocytes were paced using electrical stimulation for a maximum of 15 minutes at 2Hz with 15 Volt to cause the cardiomyocytes to contract. Using its ability to recognize repeating patterns of sarcomeres and quantify the distance between two adjacent Z-discs, the cytocypher system can track the extent of contraction based on sarcomere lengths. The cytocypher was used to measure various parameters of the cells'contractility, including baseline of sarcomere length, departure velocity, time to peak, percentage of shortening, time to baseline 70% and return velocity. Figure 10 shows one contraction together with its measured parameters.

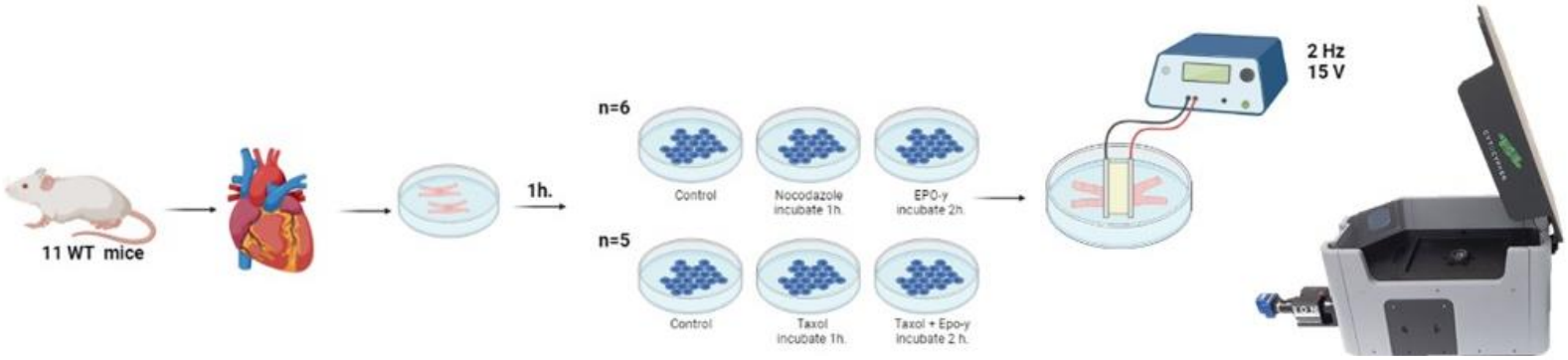


Figure 9: Cytocyper protocol. 11 WT mice hearts were isolated and their cardiomyocytes were plated in single well dishes. After one hour of plating the medium was changed to either control medium or medium containing a compound. 6 of the mice cardiomyocytes were exposed to nocodazole treatment or epo-y treatment, the other 5 mice cardiomyocytes were exposed to taxol treatment or a simultaneous treatment of taxol and epo-y. After incubation with the compound, which was 1h. for nocodozale and taxol treatment and 2 h. for epo-y and the taxol + epo-y treatment, the dishes were placed in the cytocypher device and exposed to electrical pulses using a pacer. [created using biorender®]

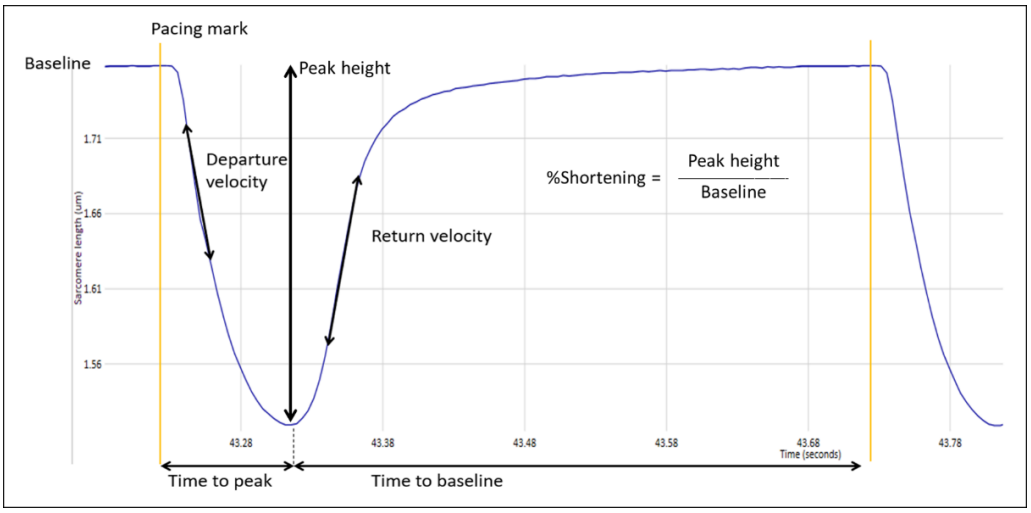


Figure 10 One cardiomyocyte contraction, measured by the cytocypher. The parameters which were measured are highlighted. These include; baseline of sarcomere length, departure velocity, time to peak, percentage of shortening, time to baseline 70%, and return velocity.

Statistics

GraphPad Prism v8 software was used for statistical analysis of the data. The Kolmogorov-Smirnov and Shapiro-Wilk tests were performed to determine the distribution of the datasets. Normally distributed data were analyzed using one-way ANOVA with Tukey's multiple comparison test. To analyze non-parametric data, the Kruskal-Wallis test with Dunn's multiple comparison test were used. The values are presented as the mean +- the standard deviation of the mean. The significance level was set at $p < 0.05$.

Results

Human tissue analysis

Limitations in tubulin polymerized and free fraction separation impairs correlation analysis with nuclear deformations

The study aims to investigate the distribution of the free and polymerized fractions over total tubulin levels in HCM myectomy tissue samples and NF donor myocardium samples, with a focus on the polymerized form that contributes to the stiffening of cells and nuclear deformations in HCM. A protocol was designed to separate the two fractions based on solubility (figure 7). GTP was added to maintain polymerization in the samples (figure 2). Western blot analysis was conducted to assess the levels of α-tubulin and its PTMs, a control sample where GTP was left out was included to confirm the presence of polymerized tubulin in the regular prepared HCM samples. To specify, in the control sample without the addition of GTP, all tubulin will depolymerize, so when GTP was left out, no tubulin should be detected in the polymerized fraction.

The expectation was to see elevation of tubulin levels in both its free and polymerized form for α-tubulin, detyrosinated, and acetylated tubulin in HCM tissue samples compared to NF donor controls. Detyrosination and acetylation are associated with stable polymerized microtubules, therefore it was expected to mainly find these proteins in the polymerized fraction sample.

The initial results in figure 11 indicate that tubulin levels are elevated in HCM samples compared to non-failing donor samples (Figure 11a-g), full blot images can be found in Appendix A. However, the no GTP control does not show what was expected for the α-tubulin and detyrosinated tubulin results, lower levels of free tubulin are detected compared to the free HCM samples. In contrast, the acetylated tubulin no GTP control did show what was expected; high levels in the free fraction and low levels in the polymerized fraction. Despite this, there was a higher increase in free detyrosinated and acetylated α-tubulin compared to the increase in the PTMs onto the polymerized fraction detected, which was unexpected, since detyrosination and acetylation mainly occur on polymerized MTs. It was suggested that a part of the polymerized tubulin ended up in the free tubulin samples, indicating that there may not have been sufficient separation of the polymerized and free fractions of tubulin. Based on these findings, the results were not considered accurate, and could not confirm sufficient separation of the polymerized and free fraction of tubulin.

To further test the protocol, a western blot experiment was conducted, as shown in Appendix B. The experiment aimed to determine the reproducibility of the protocol's results and how the total amount of tubulin was distributed between the polymerized and free forms. However, the results of the experiment did also not confirm sufficient separation of the polymerized and free forms of tubulin and thus were not regarded representative of the tubulin distribution over its free and polymerized fraction in HCM. Consequently, the tubulin analysis experiment was put on hold.

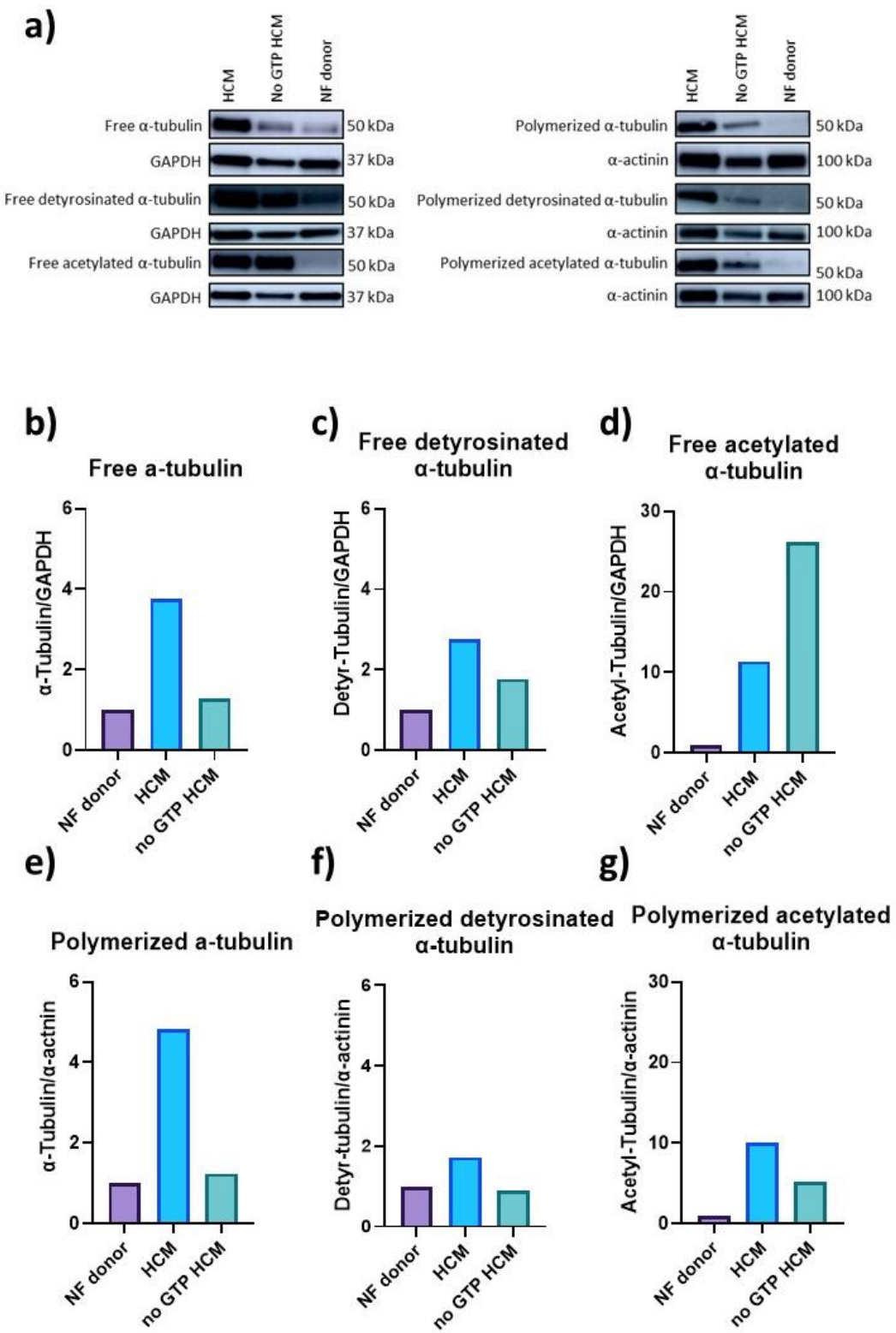


Figure 11: α-tubulin levels in HCM cardiomyocytes and its PTMs detyrosination and acetylation, normalized to NF donor protein levels. α-tubulin and its PTMs were examined in both its free and polymerized form. HCM samples show an increase of α-tubulin, as well of detyrosinated and acetylated α-tubulin compared to NF donors. a) Representative blot images for α-tubulin and its PTMs detyrosination and acetylation. b) Although unexpected, the no GTP control shows low free α-tubulin levels compared to HCM sample. c) Although unexpected, the no GTP control shows low free detyrosinated tubulin levels compared to HCM sample. d) As expected, the no GTP control shows high levels in the free form of acetylated tubulin. e) The no GTP control shows low levels of polymerized α-tubulin compared to HCM sample, as expected. f) The no GTP control shows low levels of polymerized detyrosinated α-tubulin, as expected. g) The no GTP HCM control shows low levels of polymerized acetylated α-tubulin, as expected.

Nuclear proteins lamin A/C and γH2AX elevate as a reaction to increased non-sarcomeric components

The study aimed to investigate the interaction between the nucleus and the cytoskeleton in the pathogenesis of HCM. To address subquestion 2, the levels of nuclear proteins lamin A/C and γH2AX were evaluated and correlated with nuclear parameters and non-sarcomeric components of the cardiomyocyte.

Elevated levels of lamin A/C in HCM patients is a consequence of the increase of non-sarcomeric components, leads to altered chromatin organization, and are in line with an increase in IVS thickness.

MTs and IFs are significantly increased in HCM, and in contact with lamin A/C via the LINC complex, which can alter its expression in response to mechanical stress, and is involved in chromatin organization (32). As little is known regarding the interaction between lamin A/C and non-sarcomeric components architecture in HCM development, this study utilized western blot analysis to examine lamin A/C protein levels in HCM patients in relation to NF donors and their potential correlation with nuclear parameters and non-sarcomeric components to find any possible correlations in the development of HCM.

Due to increased mechanical stress present in HCM cardiomyocytes, it was hypothesized that levels of lamin A/C would be higher in these cells compared to NF donor cardiomyocytes, as lamin A/C is known to be responsive to mechanical stress.

The western blot figures in figure 12a reveal two distinct bands – the upper band corresponds to lamin A and the lower band corresponds to lamin C. These results were evaluated through three methods: 1) by summing up the intensities of both bands to determine the total level of lamin A/C, 2) by analyzing the lamin A band separately, and 3) by analyzing the lamin C band separately.

The quantification of the blots in figure 12a and b indicate a significant increase of lamin A/C total and lamin A protein levels in HCM patients compared to the levels in NF donors ($P<0.05$). The protein levels of lamin C did not show a significant increase in HCM patients, although a slight elevation was observable. Full blots can be found in Appendix A.

Figure 12b included all 19 HCM patients (n=19), including both SMP and SMN patients. in figure 12c the SMP (n=10) and SMN (n=9) patients were analyzed separately. There was an almost significant increase in the levels of total lamin A/C and lamin A for SMP HCM patients compared to NF donors. In contrast, the levels of total lamin A/C, lamin A, and lamin C in SMN HCM patients and lamin C levels in SMP HCM patients were not significantly increased compared to the NF donors.

The protein levels of lamin A/C, lamin A, and lamin C were correlated with nuclear parameters and the levels of non-sarcomeric components in HCM patients (n=19) and nonfailing donors (n=8). There was only one correlation found for lamin A with acetylated tubulin (r=0.53). On the other hand, when correlations were analyzed separately for SMP (n=10) and SMN (n=9) patients, 16 significant correlations were observed, with r values greater than 0.5. Figure 14d shows the correlation matrices, the correlations with r-values greater than the absolute value of 0.5 are also given in XY diagrams in Appendix C. Correlations of interest were the 11 positive correlations found for both SMP and SMN lamin A/C, lamin A, and lamin C with the non-sarcomeric components (see r values in figure 12d). Moreover, the positive correlations between SMN lamin A/C, SMN lamin A, and SMN lamin C with IVS (r=0.61, r=0.50, r=0.71 respectively), and the 2 negative correlations between SMN lamin A/C and SMN

lamin C levels with the percentage area of heterochromatin in border area (r = -0.65,r = -0.86 respectively), were of interest.

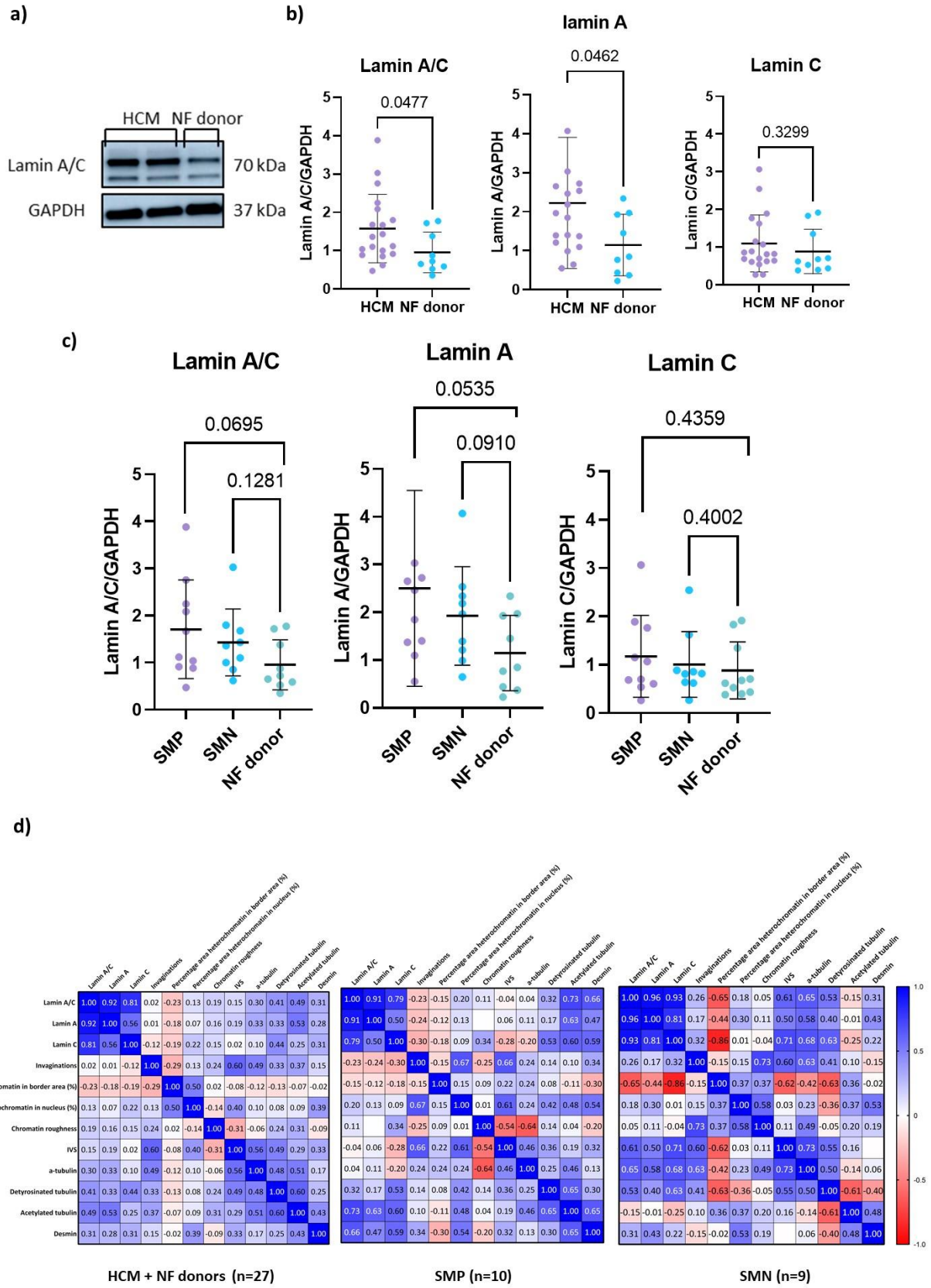


Figure 12: Lamin A/C levels in HCM cardiomyocytes and NF donor cardiomyocytes. a) Representative blot image for lamin A/C western blot,where the total amount of lamin A/C is represented by the two bands combined. The upper band corresponds to lamin A, while the lower band corresponds to lamin C. b) Significant higher levels of lamin A/C total and lamin A compared to NF donors. Higher levels visible for lamin C compared to NF donors, but not significant. c) Separate analysis of SMP and SMN HCM patients' lamin A/C levels. There is an almost significant increase of total lamin A/C and lamin A levels in SMP HCM patients compared to NF donors. d) Correlation matrices of the levels of lamin A/C and separate lamin A and lamin C as well for SMN and SMP patients.

Elevated levels of γH2AX in HCM cardiomyocytes have to do with the increase of non-sarcomeric components

As previously mentioned in the introduction, in HCM γH2AX levels are elevated. The question is, whether this increase in DNA damage is a consequence of the increased stress and stiffness of the HCM cardiomyocyte or a deficiency in DNA damage repair mechanisms. γH2AX protein levels were assessed with western blot and correlated with nuclear parameters and non-sarcomeric components in the development of HCM.

It was expected to see an elevation of γH2AX levels in HCM cardiomyocytes compared to NF donors, as HCM cardiomyocyte nuclei show alterations and are subjected to greater mechanical stress than in healthy nuclei. This stress may contribute to DNA breaks, potentially resulting in increased DNA damage.

The western blot quantification in figure 13b shows elevated γH2AX levels in HCM cardiomyocytes compared to NF donors, although not significant. Figure 13c shows the western blot analysis for the SMP and SMN patients separately, which also did not reveal any significant differences in γH2AX levels. However, there was a higher increase in γH2AX levels in SMN HCM patients compared to SMP HCM patients, when compared to NF donors.

Protein levels of γH2AX were evaluated in all HCM patients (n=19), as well as in SMN HCM patients (n=9), and SMP HCM patients (n=10), and were correlated with nuclear parameters and non-sarcomeric components. Correlation matrices can be found in figure 13d. The correlation analysis identified a total of 3 significant correlations including one negative correlation between γH2AX levels in SMN patients and acetylated tubulin (r=-0.59), and two positive correlations including one between γH2AX levels in SMP patients and detyrosinated tubulin (r=0.83), and one between γH2AX levels in all HCM patiens and detyrosinated tubulin (r=0.56).

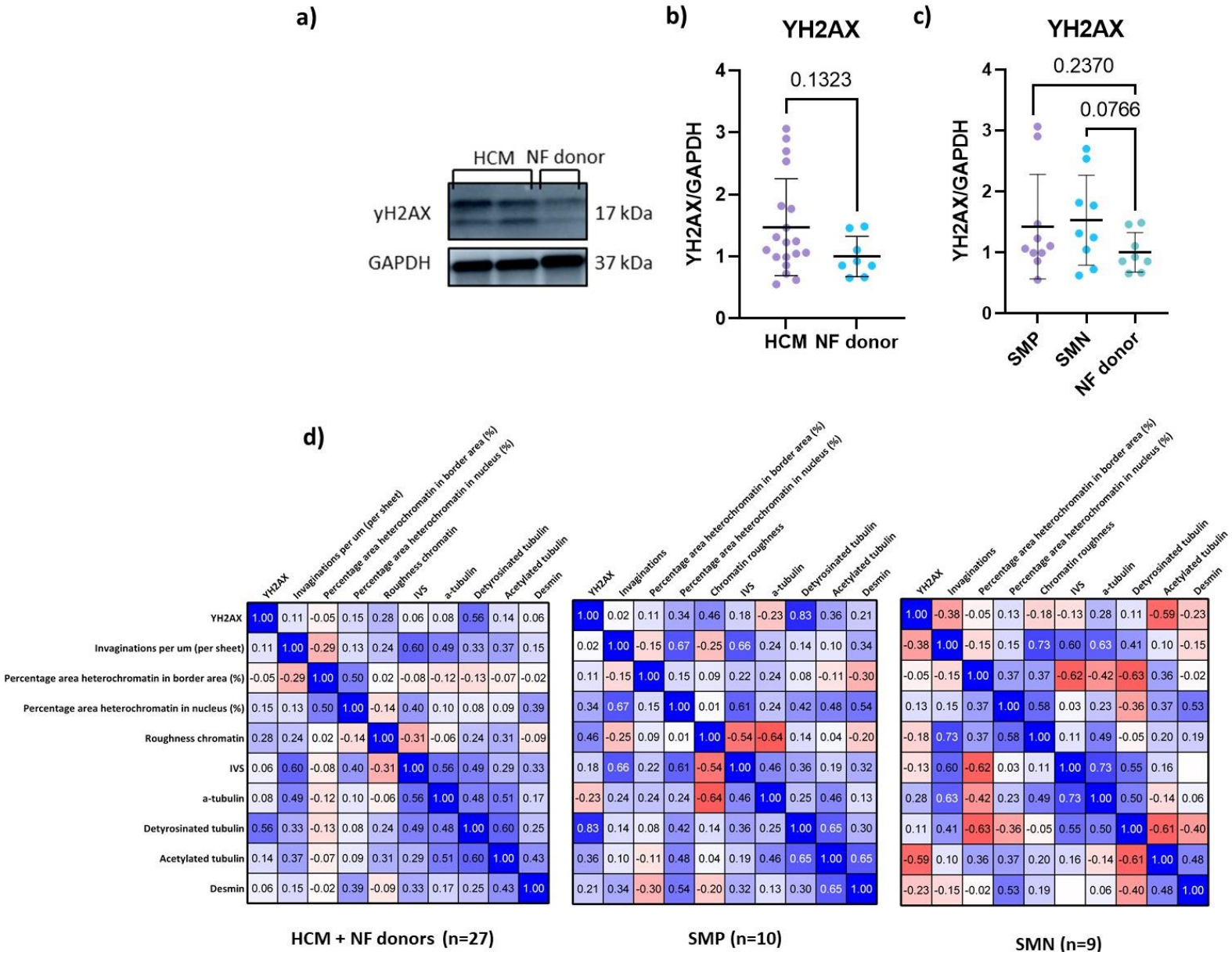


Figure 13: yH2AX levels in HCM cardiomyocytes and NF donor cardiomyocytes, and their correlation with nuclear parameters and non-sarcomeric components. a) Representative blot image for yH2AX western blot with HCM samples and NF donor samples. b) Higher levels of yH2AX visible for HCM patients compared to NF donors, however not significant. b) Separate analysis of SMP and SMN patients yH2AX levels. There is a stronger difference in yH2AX levels visible for SMN HCM patients than SMP HCM patients compared to NF donors, however not significant. d) correlation matrices for YH2AX levels in all HCM patients and donors and SMP and SMN patients separately

Mouse tissue analysis

MTs might be a potential therapeutic target in HCM, to improve the contractility of the cardiomyocyte. To investigate this possibility (subquestion 3), this study utilizes contractility measurements with the cytocypher device on isolated WT mice cardiomyocytes to which different compounds were added to manipulate microtubules and observe their impact on contractility. The results from the mice tissue analysis in this report serve as a control experiment for a larger research project examining the impact of compounds on cardiomyocyte contraction, with a specific focus on the way the nucleus deforms during contraction. Although both studies analyzed the same compounds and their resulting effects on contractillity, the larger study focused on nuclear analysis while the study in this report only focused on cardiomyocyte contractility.

Stable microtubules seem to have a supportive effect on the contraction of mice cardiomyocytes

Isolated cardiomyocytes were exposed to either control medium, or medium containing a compound including; Nocodazole, epo-y, taxol, or a simultaneous treatment of taxol and epo-y. Figure 14 and 15 present the results, which were normalized to the control group. Normalization was necessary because there were given different types of anesthesia to the mice during isolation, which affected cell contractility. However, it did not impact the observed differences between the studied treatment conditions.

It was expected that the elimination of MTs due to nocodazole treatment would lead to reduced resistance within the cell which might improve the cardiomyocyte's ability to contract and produce stronger contractions. Due to the inhibition of detyrosination after epo-y treatment, MTs might increase its dynamics, which results in less resistance, which was expected to result in improved contraction. These expectations were contrary to the obtained findings in figure 14.

Figure 14 shows an overview of the contraction parameters that were analyzed in response to treatment with nocodazole and epo-y. Compared to the control group, the nocodazole group showed a significant decrease in departure velocity (Figure 14b) and percentage of shortening (Figure 14d). Furthermore an almost significant decrease in return velocity was observerd (Figure 14f). There were no differences in the time to peak (Figure 14c) and time to baseline (Figure 14e) parameters between the nocodazole group and the control group. These findings show that contraction after nocodazole treatment is slower compared to the control, leading to impaired contraction. This observation might have to do with the almost significant increase of the baseline after nocodazole treatment compared to the control (Figure 14a). The epo-y group shows comparable results with the control group, since no significant differences were found for all parameters of the epo-y group compared with the control group.

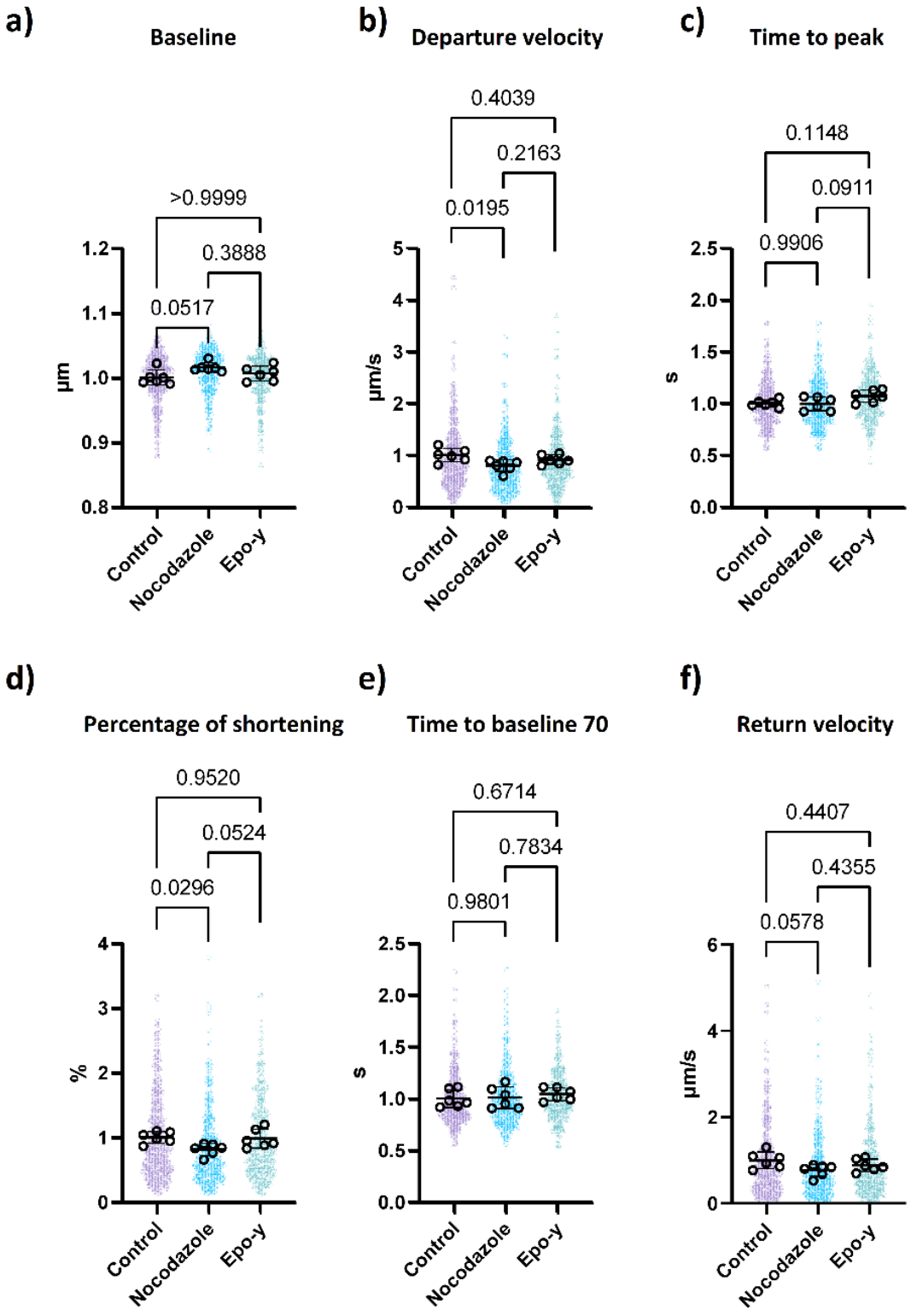


Figure 14: Contractillity parameters. To correct for differences due to the addition of different anesthesia during isolation of the heart, the results were normalized to the control group. Nocodazole and epo-y treatment effects on the contractility of mice cardiomyocytes was examined. a) Baseline. b) Departure velocity. c) Time to peak. d) Percentage of shortening. e) Time to baseline 70%. f) Return velocity. p<0.05, one way ANOVA, and post hoc tests for multiple comparisons were done. For the post hoc tests; not normal distributed samples were analyzed with Dunns multiple comparisons test (Baseline), normally distributed samples were analyzed with Tuckey's multiple comparisons test.

The 5 other mice were exposed to taxol and taxol+epo-y treatment. Taxol treatment was expected to impair contractility, due to the stabilization of MTs and increase of detyrosination, leading to less dynamic MTs, increased resistance, and therefore impaired contractility. Adding epo-y simultaneous with taxol treatment, was expected to improve MT dynamics compared to taxol alone, due to the inhibition of detyrosination, resulting in potentially better contraction. These expectations were, just like the expectations for nocodazole and epo-y treatment, contrary to the obtained findings in figure 15. Figure 15 shows an overview of the contraction parameters that were analyzed in response to treatment with taxol and simultaneous treatment with taxol and epo-y. In contrast to nocodazole, the taxol treatment showed a slight increase in percentage of shortening (Figure 15d) compared with the control, however not significant. There is a significant increase in the taxol group for the time to peak (Figure 15c), this finding shows that contraction velocity is faster, and therefore somewhat improved. Simultaneous treatment with taxol and epo-y resulted in a significant increase in the time to peak (Figure 15c) and a nearly significant increase in the percentage of shortening (Figure 15d), which indicates that contraction improves more with the combined treatment than taxol alone. No other significant differences between the control and taxol group and control and taxol + epo-y group were found.

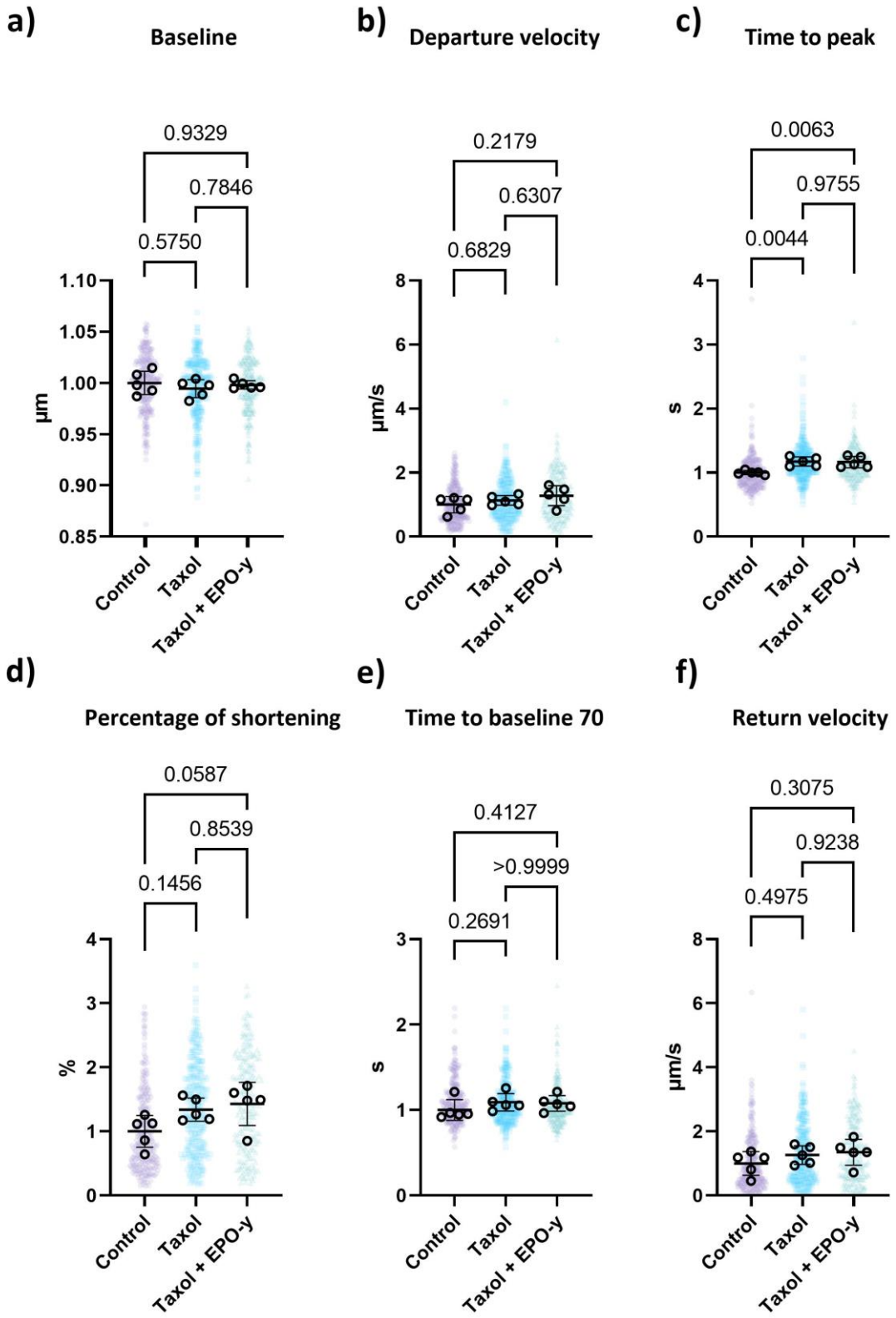


Figure 15: Contractillity parameters normalized to the control group. Examining taxol treatment and simultaneous treatment of taxol and epo-y on the contractility of mice cardiomyocytes. a) Baseline. b) Departure velocity. c) Time to peak. d) Percentage of shortening. e) Time to baseline 70%. f) Return velocity. p<0.05, one way ANOVA and post hoc tests for multiple comparisons were done. For the post hoc tests; not normal distributed samples were analyzed with Dunns multiple comparisons test (Baseline), normally distributed samples were analyzed with Tuckey's multiple comparisons test.

Discussion

Unfortunately the results of the examination of polymerized tubulin were not reliable. Nevertheless, nuclear protein and contractility analysis gave some insights into the molecular mechanism of HCM. The main findings of the study include the observation that nuclear proteins might elevate due to the increase in non-sarcomeric components, with lamin A/C increasing in an attempt to protect DNA against mechanical stress. However, insufficient lamin A/C levels and thus insufficient protection results in DNA damage, confirmed by the increase of DNA damage protein γH2AX. The study also suggested that MTs play a supportive role in cardiomyocyte contraction and inhibiting detyrosination may improve contractility even more.

Human tissue analysis

Tubulin: Polymerized and free tubulin were not sufficiently separated

The aim of the tubulin analysis was to separate the polymerized and free fraction of tubulin from frozen tissue obtained from myectomy surgery, and investigate the polymerized fraction for potential correlations with nuclear parameters. However, based on the obtained results, it cannot be ensured that there is sufficient separation of the polymerized fraction and the free fraction of tubulin. It was expected that without GTP in HCM samples, all the tubulin would have depolymerized and been present in the free fraction samples. However, there is no increase in free tubulin levels and decrease in polymerized tubulin levels in comparison to regular prepared HCM samples where GTP was added. Furthermore, in the regular HCM samples the free fraction shows a high band intensity on the western blot compared to the band intensity of the polymerized fraction. This suggests that some of the polymerized fraction may have ended up in the free fraction. These results could not be used for correlations with nuclear parameters.

The insufficient separation of the polymerized tubulin fraction and free tubulin fraction is probably mainly due to the use of frozen tissue. To clarify, myectomy samples are preserved through freezing in liquid nitrogen, to prevent degradation or alteration of its molecular structure, allowing future analysis. However, this may have affected the cell processes, leading to a decrease in biological activity (33, 34). As a result, it is very likely that most of the MTs were already depolymerized, resulting in a higher concentration of tubulin in its free fraction during the tissue analysis. In order to quantify the amounts of tubulin in its polymerized and free fraction, it is recommended to utilize fresh tissue samples, as this is more likely to result in successful separation of the two fractions. This is due to the fact that the protein cycles are still active, allowing for optimal separation between the polymerized and free tubulin (7). While freezing of the tissue is a possible explanation for the unexpected results, it is not the only factor that could have contributed to these findings. The quality of a sample can be influenced by various factors, such as temperature variations during and after surgery, temperature fluctuations during the sample preparation protocol, the conditions and duration of storage, and the type of tissue (35) . Therefore, further research is necessary to determine if the storage at -80 degrees was responsible for the unexpected results or that any other factors that play a role in sample quality may have contributed to the obtained findings.

Another method to investigate the ratio of polymerized and free tubulin might be immunofluorescent staining, which labels tubulin with a specific antibody to visualize the polymerized fraction of tubulin. In this way the polymerized tubulin can be visualized and quantified separately using fluorescence microscopy (36).

Nuclear proteins: Lamin A/C acts as a protective mechanism against increased forces applied on the nucleus in HCM

The aim of the nuclear protein investigation was to examine whether there were increased levels of lamin A/C and γH2AX proteins in HCM patients, and whether potential correlations with nuclear parameters and non-sarcomeric components could be found.

Overall, the results showed that lamin A/C is significantly increased in HCM patients compared to NF donors, in which there is a higher increase detected in SMP patients than SMN patients. In contrast, DNA damage protein γH2AX had a slight elevation in HCM patients, although not significant. However, in SMN patients, the increase in γH2AX levels was higher than in SMP patients when compared to NF donors. These findings suggest that the increase in lamin A/C may act as a protective mechanism for DNA against the increased forces applied on the nucleus, which are likely to cause DNA damage in HCM. The higher increase observed in SMP patients compared to SMN patients may contribute to the lower increase in levels of DNA damage protein γH2AX detected in SMP patients compared to SMN patients (32, 37-40). The protective function of lamin A/C against applied forces on the nucleus for DNA protection is further supported by the positive correlations between lamin A/C levels and non-sarcomeric components, which contribute to the increased forces in the cell. Additionally, the positive correlations between γH2AX and non-sarcomeric components indirectly supports this too. In particular the positive correlations observed with detyrosinated tubulin, which is a PTM known to contribute to cell stiffness and increased mechanical stress in HCM cardiomyocytes (37). Moreover, the negative correlation between SMN lamin A/C and SMN lamin C levels with the percentage area of chromatin in the border area of the nucleus provides further evidence of the protective role of lamin A/C against mechanical forces on DNA. To clarify, chromatin is more vulnerable to damage in its heterochromatin state, which is when it is densely packed (37, 41). less densely packed chromatin is more resistant to mechanical stress. It is possible that when there is less lamin A/C, the chromatin is less protected, and loosens up to become more flexible, to protect itself against stress (41). Furthermore the positive correlations found for lamin A/C, lamin A, and lamin C levels in SMN patients correlated positive with IVS, this makes sense, as the disease progresses, more hypertrophy and protein level alterations are seen.

It was also of note that in HCM lamin A was more increased than lamin C compared with NF donors. Lamin A and lamin C can independently increase or decrease. While they are both encoded by the same gene, they are produced through different splicing pathways, resulting in the production of distinct proteins (38). Therefore, factors that affect gene expression such as mRNA stability, translation efficiency, splicing, or protein stability can influence the expression levels of lamin A and C independently (42, 43). The question is whether the uneven levels of lamin A and lamin C is a result or a cause of the disease, this is not clear from the obtained results in this study and might be a subject for further research.

So, these results suggest that the increased levels of lamin A/C acts as a protective mechanism for DNA against the forces applied on the nucleus by the non-sarcomeric components.

Although this hypothesis offers a possible explanation for the observed results, it is important to note that additional evidence is needed before making definitive conclusions. Further research is required to validate this hypothesis, because this study did not investigate other potential mechanisms that could contribute to the observed differences in DNA damage protein between SMP and SMN patients, such as single stranded DNA repair mechanisms, or oxidative stress levels (44). Therefore while the findings suggest a possible relationship between lamin A/C and DNA damage protein γH2AX in HCM patients, additional research is needed to confirm this hypothesis and to identify other potential contributing factors. Other limitations were the sample size which may limit the power of the results,

a larger sample size may provide more reliable results. Also, the patients were not screened for LMNA mutations, so we cannot link the changes in lamin A/C to a possible mutation.

Mice tissue analysis: microtubules seem to support contraction in cardiomyocytes

The analysis of mice tissue aimed to investigate the effects of various compounds on cardiomyocyte contractility. Contrary to the hypothesis, the results indicate that the contractility was impaired after nocodazole treatment, but improved after taxol treatment. This suggests that MTs play a supportive role in cardiomyocyte contraction (7). One possible reason for this could be the arrangement of MTs in sarcomeres, as they are linked to the Z-discs of the sarcomeres via desmin and bear mechanical load during contraction. Deletion of MTs due to nocodazole treatment leads to the disassembly of MTs from the Z-disc, possibly causing a decrease in tension in the sarcomeres. The opposite effect might be observed after taxol treatment, which could result in an increase in tension in the sarcomeres. The expectation was that a higher tension is a limiting factor for contraction, however, the results indicate that tension applied by MTs is supportive for contraction (improved contraction was see after taxol treatment). Additionally, simultaneous treatment with taxol and epo-y showed even more improvements, which indicates that the tension applied by detyrosinated tubulin was impairing contraction (14). The results of the combined treatment do not support the hypothesis that the effect of both compounds would cancel each other out, resulting in outcomes similar to the control group. This is likely due to the incorrect hypothesis that taxol would limit contraction, which is not the case. Single epo-y treatment, which blocks detyrosination, does not seem to have an effect on the cardiomyocytes contractility. The reason why epo-y does not have an effect in single treatment may be explained by the relatively small amounts of detyrosinated tubulin present in WT mice cardiomyocytes compared to diseased cardiomyocytes (45). As a result, single epo-y treatment may not have made a significant change in the properties of MTs in the cells, but in combination with taxol (which likely also increased detyrosination) higher changes were observed. This is supported by a published study, which also observed less effects in WT samples compared to HCM samples (45). To confirm this hypothesis, it was suggested to do a protein analysis of α-tubulin and detyrosinated tubulin of the mice cardiomyocytes. Unfortunately, the lysates collected from these cells did not contain sufficient protein to conduct a western blot analysis. Therefore, the previous mentioned hypothesis that detyrosination levels were low in WT mice cardiomyocytes and higher in taxol treated WT mice cardiomyocytes could not be validated by doing a western blot to analyze tubulin and detyrosinated tubulin protein levels.

The findings from the mice tissue analysis suggest that MTs play a supportive role in contraction of cardiomyocytes and that the increase of PTM detyrosination primarily contributes to the malfunction of cardiomyocytes in HCM (7). This is further substantiated with literature studies which study detyrosination as a potential target for therapeutics in HCM (25, 45-47). While this hypothesis provides a possible explanation for the results, more evidence is definitely required before drawing conclusive assumptions. Firstly, this is because the study only utilized WT cardiomyocytes, and it may be of value to include mice cardiomyocytes with a knock in mutation of HCM to broaden the research, and study the effects of the compounds, which may show larger effects compared to the WT cardiomyocytes (45). Secondly, since the mechanisms underlying the treatment's observed effects are not yet fully understood, more research is necessary (31).

Looking at the larger picture, where the study aims to investigate the potential of microtubules as therapeutic target there are several limitations, the observed effects do not account for long-term effects on the functioning of cardiomyocytes and do also not account for off-site effects, because the treatment was performed on single ex vivo cardiomyocytes which do not take the body systems into account (31). Additionally, this study was conducted on animal tissue, which might not give accurate

results for comparing with human cardiomyocytes (31). Further research is required to determine whether the findings also apply for humans.

Conclusion

To conclude, the study had three objectives: assessing polymerized tubulin levels in HCM myectomy samples, studying correlations between nuclear protein levels and non-sarcomeric components and nuclear parameters in HCM, and determining the effect of various MT manipulating compounds on isolated mice cardiomyocytes. The results showed that the protocol used to assess polymerized tubulin levels was not reliable, furthermore lamin A/C might serve as a protective mechanism against mechanical stress on the nucleus to prevent DNA damage, and microtubules were shown to have a contributing role in the quality of cardiomyocyte contractility, with detyrosination the potential main cause for impaired contraction.

Overall, this study has the potential to contribute in the cardiac field by providing more insight on the molecular mechanisms underlying HCM, specifically focusing onto the connection between the nucleus and the cytoskeleton. This knowledge may help to identify detyrosinated tubulin as potential therapeutic target, and in the end improve patient outcomes, however it should be noted that there is still a long way to go to develop fully regenerative therapies for HCM.

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Appendix A

Protein analysis western blot membranes

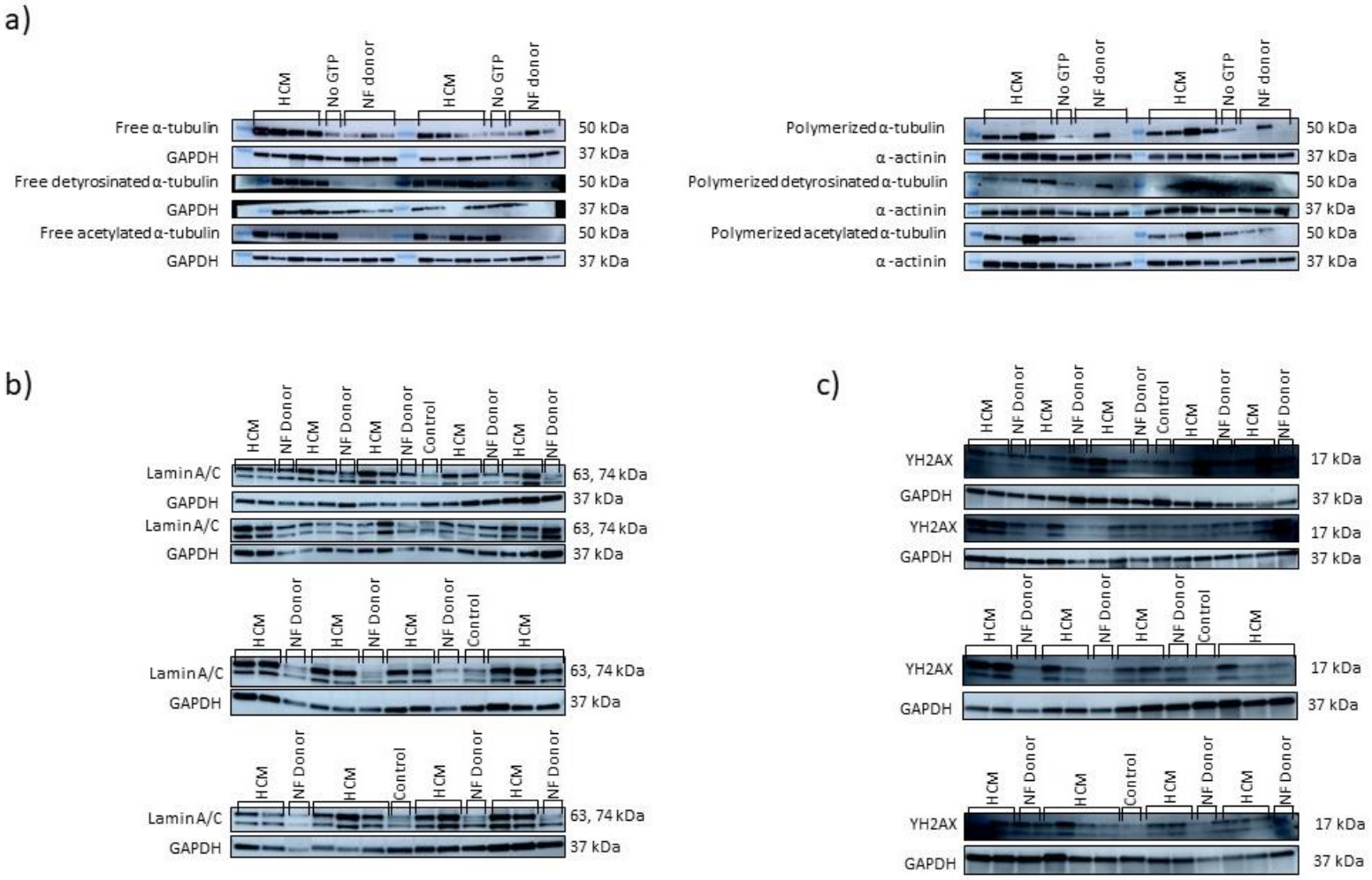

Figure 1: Western blot images of membranes for protein analysis. a) free and polymerized tubulin western blot images. b) Lamin A/C wesetern blot images. c) γH2AX western blot images.

Appendix B

An experiment was conducted to analyze α-tubulin and its PTMs in three different samples: the total sample (including both polymerized and free forms), as well as two separate samples of the polymerized and free fractions obtained by the sample preparation protocol in figure 2. Its aim was the same as that of the previous experiment: it examined how the total amount of tubulin is distributed among the free and polymerized forms. It was expected to see a clear separation between the polymerized and free fractions in relation to the overall fraction in such a way that the total fraction is about equal to the sum of the polymerized fraction and the free fraction. *Additionally in this experiment we tried various higher sample concentrations in order to obtain more dense bands on the western blot.*

The results can be found in figure 2b. The unexpected observation was that the sum of the polymerized fraction and free faction was more than the total fraction results. Therefore, the western blot didn't truly operate in the way that the results show a division of the overall fraction into the free and polymerized. When examining the no GTP HCM control, it was expected that all tubulin would be present in the free fraction. This appears to be the case, but it is difficult to draw conclusions about the effectiveness of separating the polymerized and free fractions because the results from the no GTP samples are comparable to those from regularly prepared samples.

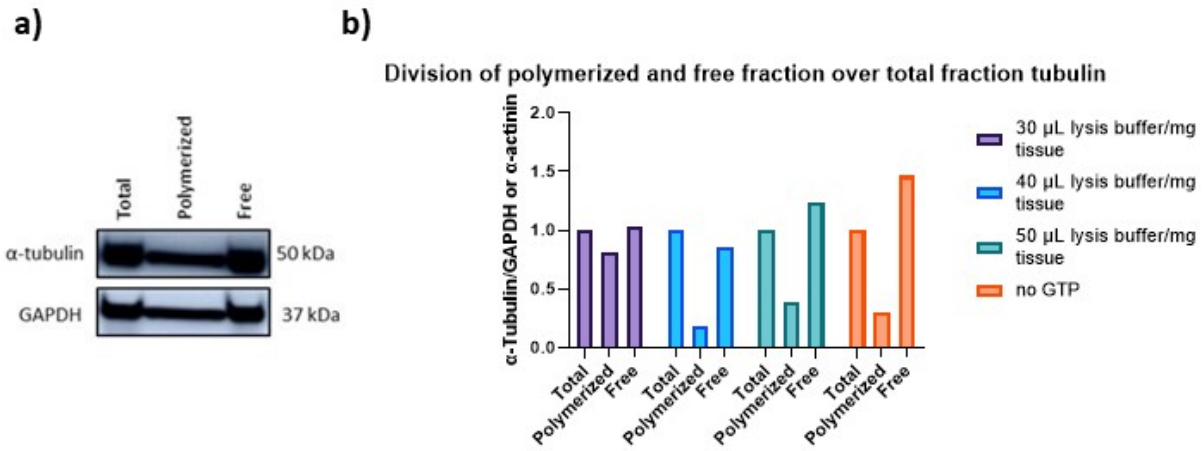

Figure 2: division of total, polymerized and free sample, normalized to the total sample. a) Representative blot image for α-tubulin in a total sample, a polymerized sample and a free sample. b) Quantificantion of western blot, the total is not equal to the sum of the polymerized and the free fraction.

The unexpected finding that the sum of the polymerized and free fraction is not equal to the total fraction may be explained by debris that is not completely filtered out throughout the protocol's centrifugation steps. Furthermore the protocol's ability to sufficiently separate the polymerized fraction from the free fraction was questioned. This is because the level of the polymerized fraction appears to be quite low compared to the free fraction tubulin levels, indicating that some of the polymerized fraction may have been misplaced in the free fraction during the sample preparation.

Appendix C

Correlations of proteins with nuclear parameters and non-sarcomeric components, with r value greater than the absolute value of 0.5.

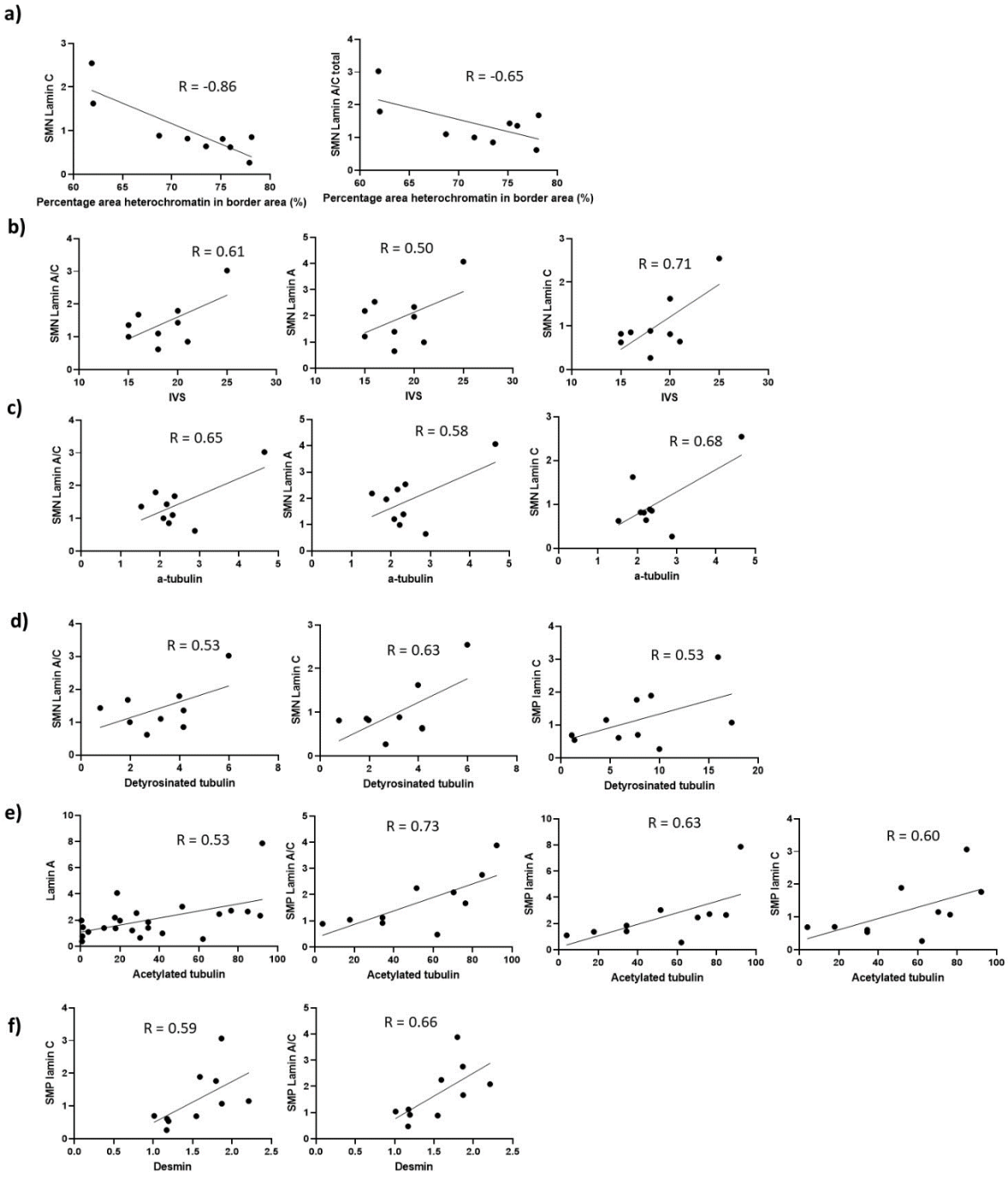

Figure 3: XY diagrams of significant correlations of lamin with nuclear parameters and non-sarcomeric components. a) Correlations with percentage area heterochromatin in border area. b) correlations with intraventricular septum thickness. c) correlations with α-tublin. d) correlations with detyrosinated tubulin. e) correlations with acetylated tubulin. f) Correlations with desmin.

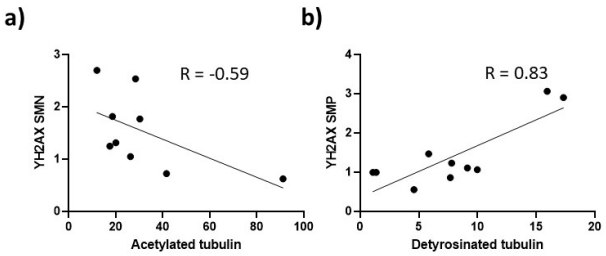

Figure 4: XY diagrams of significant correlations of γH2AX with nuclear parameters and non-sarcomeric components. a) γH2AX levels in SMN patients correlated with acetylated tubulin (r=-0.59). b) γH2AX levels in SMP patients correlated with detyrosinated tubulin (r=0.83).

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