General introduction and outline of the thesis.
Dilated cardiomyopathy

Every year, millions of people die of heart failure and in the western world it is still the number one cause of death. Worldwide 17.9 million people died of cardiovascular diseases in 2015\(^1\). In the same year, 7,667 people died of heart failure in the Netherlands and each year 40,100 people get the diagnosis of heart failure\(^2\). Heart failure is associated with various symptoms including shortness of breath, fatigue, exercise intolerance, edema and irregular heartbeats. However, heart failure is a very general term as it has multiple different etiologies. While most people associate heart failure with ischemic heart disease caused by coronary artery disease leading to myocardial infarction, there are many more cardiac diseases that cause heart failure. One of these is dilated cardiomyopathy (DCM), a cardiac disease that is characterized by enlargement of the left ventricle (LV), which coincides with thinning of the ventricular wall, and systolic dysfunction\(^3\). The latter form of cardiac remodeling is termed eccentric hypertrophy. Dilation of the ventricles results from a variety of causes such as viral infections, alcohol abuse, chemotoxicity of medicine or after a myocardial infarction. Classification guidelines indicate that DCM may be diagnosed when coronary artery disease, valvular disease, abnormal loading conditions, hypertension and congenital heart disease are ruled out as primary cause of cardiac dysfunction\(^3\). Estimates of the prevalence of DCM range from 1:2700 to 1:250\(^4,5\) which would imply that between 6,000 and 68,000 people in the Netherlands will develop DCM. Another cardiac disease in which the ventricles dilate can occur during pregnancy or within the first 5 months after birth and is called peripartum cardiomyopathy (PPCM). It is estimated that PPCM in the western world occurs in approximately 1:3000 live births\(^6\). Although patients develop the same symptoms as DCM, it seems that the cause and underlying disease mechanisms differ.

In order to understand DCM and PPCM pathogenesis, we first have to take a look at the structure of the heart muscle and mechanisms of contraction of cardiomyocytes, the cardiac muscle cells.

The structure of the cardiac muscle

The heart consists of 4 chambers divided in 2 atria and 2 ventricles. Blood enters the heart in the atria and leaves the heart through the ventricles. The right heart side receives deoxygenated blood from the body in the right atrium and after entrance into the right ventricle blood is pumped into the pulmonary arteries to be oxygenated in the lungs. The left atrium then receives oxygenated blood from the pulmonary veins and after entrance into the left ventricle, it is pumped into the aorta and oxygenated blood is delivered to the rest of the body. The pressure-volume changes during contraction and relaxation of the left ventricle are depicted in Figure 1A. A process known as passive
Figure 1: Schematic representation of a contraction and relaxation cycle of the heart.

A: Changes in pressure and volume (PV loop) of the left ventricle during a contraction and relaxation cycle. Opening of the valves are indicated with arrows, phases of the cycle with circles. B: Changes in PV loop when preload is increased. C: PV loop changes upon exercise.

filling starts when the valves between the atria and the ventricles are opened and blood can flow freely from the atria into the ventricles. This causes the ventricles to expand depending on their intrinsic compliance. The atria then contract to increase filling of the ventricles during the atrial kick. This causes a rise in intraventricular pressure and completes the filling phase, or diastolic phase, of the cardiac cycle. The ventricles contract and thereby further increase the intraventricular pressure which causes the valves between atria and the ventricles, the mitral valve (left) and tricuspid valve (right), to close preventing blood flow from the ventricles back to the atria. The phase when all valves are closed and the ventricles contract is termed the isovolumetric contraction. The rise in ventricular pressure causes the valves between the ventricles and the pulmonary artery and aorta, the pulmonary valve (right) and aortic valve (left), to open. Blood is ejected into the circulation through the pulmonary artery and the aorta. After the contraction and ejection of blood the pressure in the ventricles drops, resulting in
closures of the pulmonary and aortic valve to prevent back flow of blood from the aorta and pulmonary artery in the ventricles. The heart relaxes, but does not increase volume since both the mitral valve and aortic valve are closed, a process called isovolumetric relaxation. When pressure in the ventricles drops below the pressure in the atria, the tricuspid and mitral valves open and a new filling phase of the heart starts. The stroke volume is the amount of blood ejected in one contraction and can be calculated by subtracting the end-systolic volume from the end-diastolic volume. For appropriate function, both the diastolic and systolic phase have to be successfully completed during each contraction.

An increase in preload can occur upon increased venous return. This leads to increased diastolic volume. If the heart will eject against the same pressure this will lead to an increase in stroke volume (Figure 1B). During exercise the PV loop changes to facilitate an increase in stroke volume as well but in a different manner. Exercise also increases venous return of blood to the heart which results in increased filling of the heart. The hormone adrenaline, excreted during exercise, facilitates a more powerful contraction leading to an increase in systolic pressure and increased stroke volume, leaving the heart more depleted of blood after each contraction (Figure 1C). In heart failure, a decreased contractility leads to a decreased stroke volume. In addition, blood pressure may be increased leaving the heart to eject blood against a higher systolic pressure (Figure 2A). In dilated cardiomyopathy the ventricles are dilated leading to an increase in both end-systolic and end-diastolic volume. However, contractility is decreased leading to a decrease in stroke volume as depicted in Figure 2B.

**Figure 2:** Changes in PV loops in heart failure.
A: PV loop changes with increased afterload as occurs during high blood pressure. B: PV loop changes in DCM.
Sarcomeres: Contractile building blocks of the cardiomyocyte

Cardiac muscle is classified as a striated muscle and striations are clearly visible on electron microscopy pictures (Figure 3). These striations are due to highly organized and repetitive structures called sarcomeres. The sarcomeres run from Z-disc to Z-disc which are evident as black lines on electron microscopy images. The sarcomeres are bundled in myofibrils which are aligned with mitochondria (Figure 3). Mitochondria are small energy producing units that ensure sufficient ATP production close to the ATP-consuming sarcomeres. Sarcomeres are contractile functional units that cause the shortening of cardiac cells during contraction. Contraction occurs when the two main structures of the sarcomere, the thick and the thin filaments, slide over each other creating shortening of the whole cardiomyocyte. A schematic representation of a sarcomere and associated proteins is depicted in Figure 4. The thick filaments are predominantly composed of the protein myosin. These myosin proteins attach to the thin filaments, which predominantly consists of actin, by the formation of cross-bridges. These cross-bridges displace during a power stroke thereby moving the thin filament further over the thick filaments. This results in movement of the Z-discs towards each other thereby shortening the cell (Figure 4).
Excitation-contraction coupling and Ca\(^{2+}\) cycling

Contraction starts with an action potential generated in pacemaker cells in the heart. Depolarization of the membrane induced by the action potential causes opening and closure of various ion channels in cardiomyocytes. Ca\(^{2+}\) is the most important ion for the regulation of contraction. The sarcoplasmic reticulum (SR) of the cardiac cells is important for the cycling of Ca\(^{2+}\) during contraction. A large pool of Ca\(^{2+}\) is stored in the SR and released upon a process called Ca\(^{2+}\)-induced Ca\(^{2+}\)-release. Depolarization of the cardiac cells during an action potential causes Ca\(^{2+}\) to enter the cells through the L-type Ca\(^{2+}\) channel. This Ca\(^{2+}\) can trigger release of Ca\(^{2+}\) from the SR through the ryanodine receptor. The Ca\(^{2+}\) then binds to the troponin complex in order to facilitate contraction. The troponin complex consists of three different troponin proteins; cardiac troponin T (cTnT), cardiac troponin I (cTnI) and cardiac troponin C (cTnC). Together with tropomyosin, the troponin complex is important for the regulation of force generation.
and Ca\textsuperscript{2+}-sensitivity of myofilaments\textsuperscript{8}. A schematic representation of the troponin complex and induced conformational changes upon binding of Ca\textsuperscript{2+} are depicted in Figure 5. cTnI inhibits actin-myosin interaction and plays an important role in the Ca\textsuperscript{2+}-sensitivity of myofilament activation through its interaction with cTnC\textsuperscript{9}. The troponin complex can lock tropomyosin in a position where it sterically hinders the interaction between myosin and actin at low Ca\textsuperscript{2+} concentrations to prevent contraction during diastole. During contraction Ca\textsuperscript{2+} binds to cTnC which results in a large conformational change in cTnI which leads to displacement of its inhibitory domains away from actin and thereby releasing its inhibitory effect on actin-myosin interaction\textsuperscript{10, 11}. Tropomyosin moves which enables myosin to bind to actin and subsequently cause force generation. The final shift of tropomyosin after myosin has bound facilitates the formation of a strong cross-bridge. By binding to both cTnC and tropomyosin\textsuperscript{12}, cTnT regulates ATPase activity during contraction, but also serves as an anchor on the thin filaments for the troponin complex. In order to facilitate diastole and appropriate filling of the heart muscle, the cytosolic Ca\textsuperscript{2+} needs to be removed. The protein SERCA pumps Ca\textsuperscript{2+} back into the SR thereby restoring the Ca\textsuperscript{2+} buffer in the SR for the next contraction. The protein phospholamban (PLB) is an inhibitor of SERCA and interaction between PLB and SERCA can fine tune the amount of Ca\textsuperscript{2+} pumped back into the SR. Therefore, a reduction of available Ca\textsuperscript{2+} during contraction, or increased levels of Ca\textsuperscript{2+} when the cardiomyocytes need to relax, can have significant implications for both systolic and diastolic function of the heart. In heart failure this cycling of Ca\textsuperscript{2+} is impaired\textsuperscript{13, 14}. Leakage of Ca\textsuperscript{2+} from the SR due to leaking ryanodine receptors in heart failure causes increased diastolic Ca\textsuperscript{2+} levels\textsuperscript{13, 15}. In addition, SERCA is downregulated in various forms of heart failure leading to reduced reuptake of Ca\textsuperscript{2+} in to the SR\textsuperscript{14, 15}. This raises diastolic Ca\textsuperscript{2+} levels during diastole and limits the amount of Ca\textsuperscript{2+} available to be released from the SR for the next contraction. The increased levels of intracellular Ca\textsuperscript{2+} can also induce the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger to exchange Ca\textsuperscript{2+} for Na\textsuperscript{+}. This can lead to partial depolarization and might induce arrhythmias\textsuperscript{13-15}. Cardiomyocytes can also alter their myofilament sensitivity for Ca\textsuperscript{2+} in order to fine-tune function. This is mostly done through phosphorylation of cTnI.

**The β-adrenergic system and contractility**

The heart is able to adjust the contraction cycle in various ways in order to fine-tune the amount of blood circulating the body. One of the well-known adaptive processes is induced by a chemical called adrenaline. Adrenaline is known to increase heart rate and thereby cardiac output which is the product of stroke volume and heart rate. Apart from the effect of adrenaline on heart rate, adrenaline also causes the activation of protein kinase A (PKA) through stimulation of the β-adrenergic receptors on cardiomyocytes. PKA is able to phosphorylate various proteins thereby altering their function. One of
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Figure 5: Schematic representation of the troponin complex.

The troponin complex consists of cTnI (blue), cTnT (yellow) and cTnC (red). Upon binding of Ca\(^{2+}\) the complex undergoes a conformational change in which cTnI moves away from actin and tropomyosin (purple) moves which allow myosin to bind actin and generate force. The letter H indicates a helical structure, IR indicates the inhibitory region. N and C indicate the N-terminus and C-terminus respectively of the proteins.

Figure adapted from Bollen et al. (2017) Journal of Physiology.

these proteins is cTnI. PKA-mediated phosphorylation of cTnI results in a decreased myofilament Ca\(^{2+}\)-sensitivity and Ca\(^{2+}\) is released quicker by the myofilaments. Combined, these changes allow for proper relaxation which is important for diastolic function. In addition, phosphorylation of PLB relieves its inhibitory effect on SERCA which enables a faster re-uptake of Ca\(^{2+}\) in the SR ensuring sufficient Ca\(^{2+}\) is available for the next contraction.

Since the stroke volume is often decreased in heart failure due to defects in the cardiac muscle, the body tries to compensate by increasing heart rate in order to maintain cardiac output. However, chronic overstimulation of the β-adrenergic receptor system causes desensitization of the β-adrenergic receptors causing a limited response to adrenaline which will further impair cardiac pump function\(^1\). Many heart failure patients receive β-blockers in order to limit harmful effects of the chronic overstimulation of the
β-adrenergic system. Changes in myofilament Ca$^{2+}$-sensitivity can have pronounced effects on cardiac function. Increased myofilament Ca$^{2+}$-sensitivity can compensate for the depletion of Ca$^{2+}$ in heart failure by inducing stronger contraction at low Ca$^{2+}$ concentrations. However, it may also cause contractions in conditions of low Ca$^{2+}$ during diastole leading to impaired filling or cardiac arrhythmias. On the other hand, decreased myofilament Ca$^{2+}$-sensitivity can decrease systolic function by producing less tension at high Ca$^{2+}$ during systole. The weaker contraction will result in decreased stroke volume and subsequent systolic dysfunction. Maintaining appropriate Ca$^{2+}$-sensitivity of myofilaments is therefore important for both systole and diastole. A schematic representation of a force-Ca$^{2+}$ curve is depicted in Figure 6 to illustrate changes in contractility in systole and diastole when myofilament Ca$^{2+}$-sensitivity is altered by the β-adrenergic system.

Figure 6: Changes in Ca$^{2+}$-sensitivity due to β-adrenergic stimulation.

Upon β-adrenergic stimulation the force-Ca$^{2+}$ curve shifts to the right which leads to a decreased Ca$^{2+}$-sensitivity and decreased force development at a given [Ca$^{2+}$] to ensure proper relaxation. In situations of desensitized β-adrenergic receptors as occurs in heart failure, the force-Ca$^{2+}$ curve shifts to the left which leads to an increased Ca$^{2+}$-sensitivity and increased force development at a given [Ca$^{2+}$] to ensure sufficient contractile power. However, this may also lead to inappropriate contractions, or impaired relaxation, in diastole when [Ca$^{2+}$] is low.

Length-dependent activation of myofilaments

In addition to activation of the beta-adrenergic system, increased stretching of the heart enhances cardiac pump function during increased demand as occurs during exercise. An increase in venous return causes increased diastolic ventricular filling and therefore increased stretch of the cardiomyocytes within the heart. The heart responds to this by a stronger contraction and therefore generates a larger stroke volume to match cardiac output with the increased cardiac input as previously described. This principle is
called the Frank-Starling mechanism and the cellular basis involves length-dependent activation of myofilaments. Length-dependent activation of myofilaments depends on an increase in Ca\(^{2+}\)-sensitivity and maximal force development upon stretch. This principle can be visualized in the laboratory by measuring the force-Ca\(^{2+}\) relation at different sarcomere lengths in single membrane-permeabilized cardiomyocytes. We determine the \([\text{Ca}^{2+}]\) needed to achieve 50% of maximal force (EC\(_{50}\)). The difference in EC\(_{50}\) at both sarcomere lengths, ΔEC\(_{50}\), is a measure of length-dependent activation (Figure 7B). Upon stretch, the maximal force increases but also the force-Ca\(^{2+}\) relationship shifts to the left (Figure 7A). Length-dependent activation is influenced by cTnI and titin. An increase in compliant titin and hypophosphorylation of cTnI have been shown to impair length-dependent activation.

**Figure 7:** Length-dependent activation of myofilaments.

A: The force-Ca\(^{2+}\) curve shifts upwards and to the left upon stretch of a cardiomyocyte. This causes an increased Ca\(^{2+}\)-sensitivity and increased maximal force development of myofilaments at a higher sarcomere length compared to a shorter sarcomere length. B: In the laboratory we assess length-dependent activation by measuring the shift in Ca\(^{2+}\)-sensitivity and express it as ΔEC\(_{50}\) in single cardiomyocytes.

**Titin and active and passive force development**

As mentioned above, the protein titin can affect length-dependent activation of myofilaments. Titin is a giant sarcomeric protein and acts as a spring running all the way through the sarcomeres from the Z-disc to the M-line (Figure 3). Titin can exist as 2 different isoforms; stiff N2B or compliant N2BA titin. Both isoforms coexist in the human heart and their ratio is important for the compliance of the heart muscle via modulating passive tension of cardiomyocytes. N2BA leads to a more compliant heart muscle that is more able to fill in diastole compared to a heart with predominantly N2B titin.
However, it also believed that the compliance in titin is important for the preload of the heart. More stiff titin would theoretically cause a higher strain on the heart upon filling and can thereby increase contractility. An increase in compliant titin has been associated with a decreased length-dependent activation\(^\text{19}\). However, the exact mechanisms by which titin can affect systolic function are largely unknown and disputed. Apart from a switch in titin isoform composition, post-translational modification of titin can also alter passive tension of cardiomyocytes. While phosphorylation in the N2B unique sequence by predominantly PKA can reduce passive tension, phosphorylation of the PEVK domain by protein kinase C (PKC) or CaMKIIδ can increase passive tension\(^\text{22, 23}\). Therefore, the impaired β-adrenergic signaling pathway in heart failure as mentioned above can cause PKA-mediated hypophosphorylation of titin and thereby increase passive tension and impair diastolic function.

The changes in cTnI phosphorylation and titin isoform composition that occur in heart failure are believed to be part of secondary disease remodeling. Secondary disease remodeling are changes that occur in heart failure in response to altered cardiac demands and represent general hallmarks of the disease. Secondary disease remodeling can be pathological and thereby contribute to disease progression. However, secondary disease remodeling can also be induced in order to cope with the altered cardiac demand and thereby delay severe cardiac dysfunction.

**Genetic predisposition of dilated cardiomyopathy**

Apart from secondary disease remodeling in DCM, mutations in genes can contribute to disease pathogenesis and even be the cause of DCM. With current genetic screening techniques, a mutation can be identified in 20-50% of DCM patients\(^\text{24-26}\). These mutations have been found in more than 30 different genes\(^4\). Most of the affected genes (\(TTN\), \(TNNT2\), \(TNNT3\), \(TNNC1\), \(TPM1\) or \(MYH7\)) encode for the sarcomeric proteins titin, cTnT, cTnI, cTnC, tropomyosin and myosin heavy chain respectively, and may thus directly affect cardiomyocyte function. However, also mutations in genes encoding nuclear envelope proteins such as lamin A/C (encoded by \(LMNA\)) or cytoskeletal proteins such as desmin (encoded by \(DES\)) are found in DCM patients, which implies that alterations in cellular structure in general lead to DCM. Although mutations in genes encoding proteins of the sarcomeres, nuclear envelop and cytoskeleton are likely to induce DCM via different mechanisms based on their diverse intrinsic properties, they all lead to the same clinical phenotype. Alternatively, certain mutations may rather act as a disease modifier and contribute to a poor prognosis by aggravating cellular dysfunction. Therefore, identified DCM mutations may determine age of disease onset, prognosis, treatment and family counseling. However, although multiple DCM mutations have been identified, currently
treatment of DCM is similar for all patients independent of underlying mutation, and is mostly based on clinical symptoms. More in depth knowledge about the mechanisms behind the disease initiation and progression of DCM in general but also genotype-specific alterations in heart function is warranted to ultimately come to patient-specific counseling.

Aim and outline of this thesis

The patient population of DCM is very heterogeneous as well as the underlying causes. In this thesis we define general pathogenesis and secondary cardiac remodeling in DCM. We studied different patient populations ranging from pregnant women with DCM to pediatric DCM and adult onset DCM. Pregnant women who develop PPCM can show rapid cardiac deterioration during or soon after pregnancy. PPCM was believed to be DCM triggered by the increased hemodynamic load during pregnancy in the past. However, the peak of disease onset in PPCM does not coincide with the increase in hemodynamic load. In addition, a key role for the hormone prolactin, a hormone largely absent in non-pregnant and non-nursing individuals, in PPCM pathogenesis has been shown. In light of these findings DCM and PPCM are now considered two different diseases. However, the overlap and deviations in underlying pathomechanisms are largely unknown. Chapter 2 gives an overview about the differences and similarities between DCM and PPCM. In chapter 3 we assessed the cellular differences in a head to head comparison between DCM, PPCM and ischemic heart disease. Chapter 4 focuses on pediatric DCM patients who have such severe heart failure that they needed a cardiac transplantation already in childhood. We explored if the pediatric DCM patients harbor the same pathogenesis as we observed in the previous chapters in adult DCM patients.

We also studied heart tissue of DCM patients with various mutations to study genotype-specific pathogenic effects. In chapter 5 a DCM patient is described who was explanted at the age of 19 years old and carried a mutation in the gene RBM20, a splicing regulator of titin, in order to determine specific pathogenic changes induced by this mutation. In chapter 6 a large cohort of patients carrying the $\text{LMNA}_{p.R331Q}$ mutation was clinically evaluated and the cellular changes induced by this mutation unraveled. In chapter 7 patients with the non-sarcomeric $\text{LMNA}_{p.R331Q}$ mutation were compared to patients carrying the sarcomeric mutations $\text{TNNT2}_{p.K217 deletion}$ and $\text{TNNI3}_{98 truncation}$. This chapter aimed to distinguish between secondary disease remodeling and genotype-specific pathogenic effects. In chapter 8 the findings throughout this thesis are discussed and we classify our experimental findings into cellular changes that are primary disease-causing or secondary to disease remodeling.
References


