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Proefschriften

The path of life of cardiac troponin T
Proteomic analysis of circulating proteoforms

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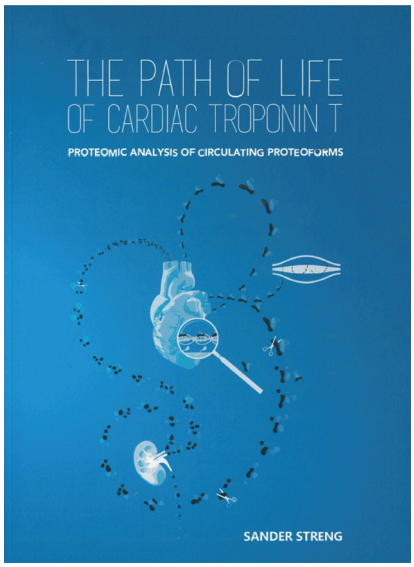
Acute myocardial infarction and cardiac troponins

Acute myocardial infarction

Despite a strong decline in cardiovascular related mortality since 1980, cardiovascular disease still accounted for 28% of total deaths in the Netherlands in 2014 and 37% worldwide (1, 2). A significant part of these deaths (23%) was a result of ischemic heart disease including acute myocardial infarction (AMI), commonly known as a "heart attack" (1). AMI is defined as myocardial cell death due to prolonged ischemia resulting from an acute imbalance between oxygen supply and demand (3). The rupture or erosion of a coronary atherosclerotic plaque and the subsequent blocking of downstream arteries is the most common cause of AMI (4). Oxygen, glucose, and nutrients transported through the bloodstream are then denied access to the affected region of the heart, resulting in cardiomyocyte apoptosis and necrosis within minutes of the event (5, 6). Depending on the size of the affected area, (severe) impairment of myocardial pump function due to cardiomyocyte loss can lead to chest discomfort, pain, disability, loss of consciousness, and death.

The rapid diagnosis of AMI is of paramount importance to a timely and accurate therapeutic response (7). As the clinical symptoms associated with AMI (e.g. chest pain, dyspnea, nausea, diaphoresis, and fatigue) are non-specific, additional diagnostic techniques are required for an accurate diagnosis. The 12-lead electrocardiogram (ECG) is the first diagnostic tool used in an emergency setting and is recommended to be obtained within 10 minutes after the arrival of the patient or, preferably, in a preclinical setting (e.g. the ambulance) (8). Persistent (>20 minutes) ST-segment elevations on the ECG, in combination with typical clinical symptoms, generally reflect a total coronary artery occlusion and will likely develop to an ST-elevation myocardial infarction (STEMI) (9). In these cases, immediate treatment is indicated (10). Less clear are cases in which patients present themselves with chest pain, but without these ST-elevations. These patients may be diagnosed with non-ST-elevation myocardial infarction (NSTEMI) when cardiomyocyte necrosis can be determined, or as unstable angina (UA) when cardiomyocyte ischemia has not yet resulted in cell death (11). A number of non-cardiac related conditions are also possible (7, 12). In order to definitely diagnose a patient with AMI (both STEMI and NSTEMI), it follows from the definition of a myocardial infarction that it is necessary to determine whether or not myocardial cell death has occurred. Historically, the concentration of cardiac cytosolic proteins (like aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatine kinase MB isoenzyme (CK-MB), and myoglobin) released upon cardiomyocyte necrosis was determined in patient serum (13). However, these markers are not specific to cardiac tissue and are widely expressed in other tissues like the liver, kidneys, and skeletal muscle (14). New, non-protein, markers currently under investigation are cardiac-specific micro-RNAs, possibly playing a role in the future (15). According to the current guidelines, the preferred way to determine myocardial cell death



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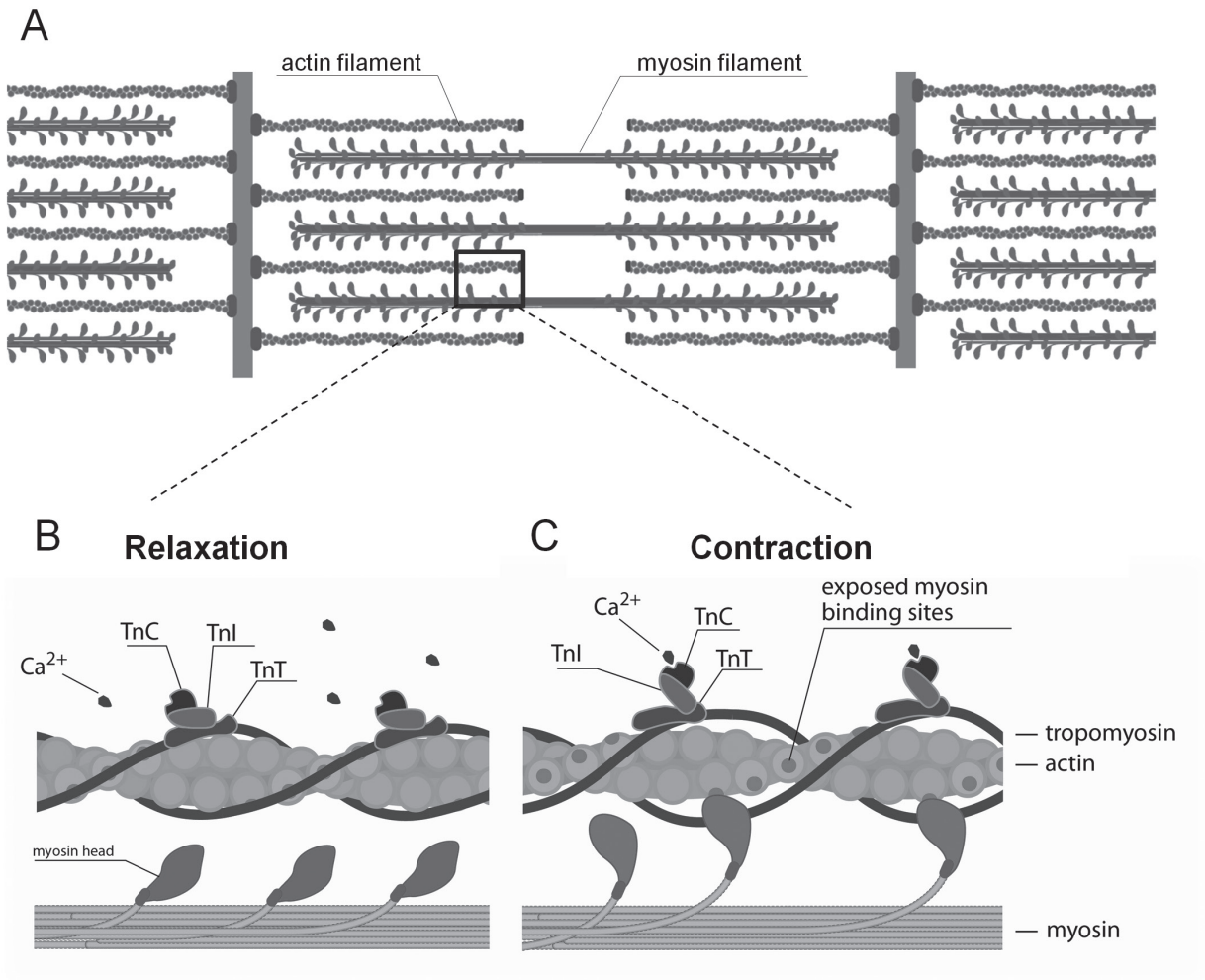

Figure 1. A. Schematic of the sarcomere containing the actin (thin) filaments and the myosin (thick) filaments. **B.** In the relaxed state, tropomyosin blocks access to the myosin binding sites on the actin molecules. **C.** During muscle contraction, Ca^{2+} binds to troponin C (TnC) resulting in a conformational change in troponin I (TnI) and T (TnT). Tropomyosin slides away, allowing myosin to bind to actin. The sarcomeres shorten, resulting in muscle contraction. Figure republished from (21), with permission.

is by measuring the rise or fall in either serum or plasma concentration of cardiac troponins during a one or three hour window (3, 11). Because of their unique cardiac specificity, these proteins are being used as the biomarkers of first choice for the detection of cardiac injury.

Cardiac troponins

Cardiac troponin I (cTnI) and T (cTnT) are proteins involved in the regulation of cardiac muscle contraction and are expressed solely in the heart. Together with the non-cardiospecific troponin C (TnC), these three proteins form a ternary protein complex situated on the thin filaments of cardiomyocytes (16). The thin and thick filaments together form the sarcomere; the smallest contractible unit of a myofibril, which is itself the smallest unit of a muscle fibre. While the thick filaments consist mainly of myosin, the thin filaments consist of a repeating polymer of 7 actin monomers, held together by tropomyosin (Tm). The cardiac troponin (cTn) complex is anchored on top of Tm by cTnT (17-19). Because of the elongated and rigid structure of cTnT, any conformational change resulting in a force acting upon it is transferred to Tm; essential for the mechanism of muscle contraction.

Muscle contraction is initiated with an action potential: a rhythmic electrical signal that originates from the Sinoatrial (SA) node and propagates via the bundle of His and the Purkunje fibres throughout the heart. This action potential depolarizes the cell membranes of cardiomyocytes, leading to a massive and sudden influx of Ca^{2+}-ions increasing the intracellular Ca^{2+}-concentration. In this concentration, Ca^{2+} is allowed to bind to TnC, resulting in a cascade of conformational changes in the cTn complex. In this cascade, cTnI can be thought of as the "lever" that pushes Tm away from the myosin binding sites on the actin molecules that were previously hidden underneath. Myosin is then able to interact with the exposed binding sites and initiates muscle contraction by consuming an ATP molecule which fuels the resulting power stroke (**figure 1**). When the action potential has passed, the cell membranes repolarize and Ca^{2+} is transported out of the cell. Myosin and actin dissociate, the conformational changes revert, and the muscle relaxes. This cycle of contraction and relaxation lasts about 800 milliseconds at rest and repeats itself constantly, for as long as we live.

The "path of life" of cardiac troponin T

The "path of life", or lifespan, of a single cTnT (or cTnI) molecule can be divided into two distinct phases which I will call the "physiological lifespan" and the "clinical lifespan". The physiological lifespan can be defined as the phase that begins with the synthesis of cTnT in the ribosomes of cardiomyocytes and ends with the release of cTnT from these cells into the blood circulation. Consequently, the clinical lifespan begins with the release of cTnT into the circulation and ends with the removal of cTnT from the blood circulation by one of two possible mechanisms.

The physiological path of life

In the human genome, three genes encode for troponin T. These genes are TNNT1 (coding for slow skeletal troponin T; ssTnT), TNNT2 (coding for cardiac troponin T; cTnT), and TNNT3 (coding for fast skeletal troponin T; fsTnT) (20, 21). The physiological path of life for cTnT starts with the process of transcription and translation of the TNNT2 gene to form one of 12 possible cTnT isoforms, of which the healthy human adult form (isoform 6, cTnT3) is the most abundant.

Within the cardiomyocyte, cTnT is bound primarily to the myofibrils, but a small percentage (6-8%) was originally thought to be present as an unbound protein freely in the cytosol (22-24). However, others have found that 0% or even 80% of the cTn is present as free, unbound protein (25, 26). Recent work by Starnberg and colleagues suggests, however, that there is no fixed amount of cTnT present in the cytosol, but that the bound and unbound fractions vary based on their respective concentrations, trying to maintain an equilibrium (27). It is possible that this reflects some kind of tuning mechanism to increase or decrease the amount of cTnT bound to the myofibrils. Another such tuning mechanism is the presence of posttranslational modifications that may temporarily alter the structure and function of cTnT and other proteins (21, 28). These modifications (mainly phosphorylation and degradation) can either be the result of a regulatory process in order to actively fine tune cardiac output (such as is the case with protein kinase A (PKA) mediated phosphorylation of cTnI during strenuous exercise (28-30)), or a pathologically induced effect (such as the hypothesized protein kinase C (PKC) mediated phosphorylation of cTnT in heart failure) (31, 32). The irreversible degradation of the highly acidic N-terminus of cTnT is also thought to influence cardiac contraction and is modulated by PKC phosphorylation. In the final stages of the physiological lifespan, when either apoptosis or necrosis is imminent, the cTns, and the cardiomyocyte as a whole, are subject to proteolytic degradation by caspases and calpains (33-36). When the cell dies, cTnT has completed its physiological path of life, and an entirely new phase is about to begin.

Cardiac troponin's clinical path of life

The clinical path of life starts with the release of cTnT into the blood circulation. At that point, the released cTnT-molecule has no longer a physiological function, but its presence in the circulation can be of importance to clinicians when diagnosing a patient. If the underlying reason of troponin release is an AMI, the release kinetics of cTnT and cTnI are initially highly similar (**figure 2**). After reaching their apex at approximately 24 hours, the serum co33-36ncentration of cTnI gradually decreases, while the concentration of cTnT remains elevated for several days before decreasing. This "biphasic release pattern" has originally been subscribed to the initial release of cytosolic cTnT followed by the slow washout of structurally bound cTnT (22, 23), but this hypothesis is now uncertain in light of the recent findings by Starnberg as discussed in the previous section (27). Also unclear, however, is why this release pattern is absent for cTnI.

When the measured serum concentration of cTn in a patient with symptoms of myocardial ischemia exceeds the 99th percentile of a healthy reference population (in the case of cTnT: 14 ng/L, **figure 2**, dotted line (37)), the diagnosis of AMI can be confirmed (3, 11). The advent of high-sensitivity cardiac troponin assays (which by their definition are able to detect cTn in >50% of the healthy population (38)) has led to an increase in sensitivity to near 100% (39, 40). However, this increase in sensitivity came at the cost of specificity and resulted in the detection of cTn elevations in the absence of AMI (41-43). Examples of these are cTn elevations in patients suffering from end-stage renal disease (ESRD) (44-47), patients suffering from type 2 diabetes mellitus (48), healthy endurance athletes (49-52), and the elderly (53, 54). Although each elevation of cTnT or cTnI is associated with a worse prognosis and an increa se in morbidity and mortality (39, 55-57), it is as of yet unclear if all of these elevations represent irreversible cardiomyocyte necrosis or if part of them can also be subscribed to a specific physiologic release or leakage of cTn from otherwise viable cardiomyocytes (58, 59).

Regardless of the underlying mechanism of cTn release, the majority of its clinical lifespan cTnT spends circulating through the bloodstream. This new environment is a hazardous place as macrophages, immunoglobulins, and proteolytic enzymes are present in high abundance and interact with the foreign cTnT and cTnI molecules in ways that did not occur previously. As a result of this, both cTnT and cTnI have been found progressively degraded in patient serum as a function of time since the onset of AMI (60-63). Where cTnI is concerned these findings were uniformly accepted decades ago and are thought to be in part responsible for the lack of standardization of the many clinical cTnI assays on the market (60, 64-66). However, for cTnT, the fragmentation hypothesis met with strong opposition as opponents of the theory argued that its presence in serum was mainly in the intact form and in complex with cTnI and TnC (67, 68). Even though the arguments of the opponents were recently disproved (69), this ongoing debate is at the basis of a major part of this thesis. Other alterations to the structure of cTnT have also been suggested to occur in the circulation, including phosphorylation (70), ubiquitination (possibly preceding its degradation) (71), complex-formation such as macro-troponins (72) and the binding of cTnT with specific anti-cTnT immunoglobulins (73, 74).

The cTnT present in a serum sample of each individual patient consists of a heterogeneous mixture of all of these different molecular forms of cTnT. Moreover, this mixture does not stay constant, but varies as time goes on and as different sequential serum samples are collected. This is problematic because the clinical assay is tuned and calibrated for intact, recombinant, cTnT (hs-cTnT package insert, Roche diagnostics) which does not correspond to this highly variable and heterogeneous in vivo situation. The antibodies of the clinical assay may have a different affinity for these different molecular forms of cTnT, possibly leading to an over- or underestimation of the "true" cTnT concentration. Interestingly, this may also be the explanation for the biphasic release mechanism shown in **figure 2**. In addition, some of these forms may only be created in specific cases or under specific conditions. If so, then determining these specific molecular forms can aid in differentiating patients with similar elevated cTnT values.
Eventually (in the vast majority of cases), the cTnT (and cTnI) concentrations return to below the reference value. When a single cTnT molecule is removed from the circulation or if it is degraded to the point that it is no longer recognisable by the clinical assay, its 'life' has come to an end. However, it is not yet fully understood how exactly cTnT leaves the blood circulation. One possible explanation is that it is being degraded completely in the circulation and that its individual amino acids are recycled to form other proteins. The other explanation is that cTnT is cleared by the kidneys. It has been suggested that degradation is first needed before selected fragments can be cleared by the kidneys and that the observed cTnT elevations in ESRD patients are caused by a reduced clearance (75). A combination of both hypotheses is also a possibility.

Proteomics and mass spectrometry
The majority of the work described in this thesis is the result of different proteomics workflows. The field of proteomics is the study of the structure and function of proteins (76). Proteins, like cTnT, fulfil a myriad of functions in the cells that make up our body and unravelling the different pathways and processes that drive key functions is the main goal of proteomics

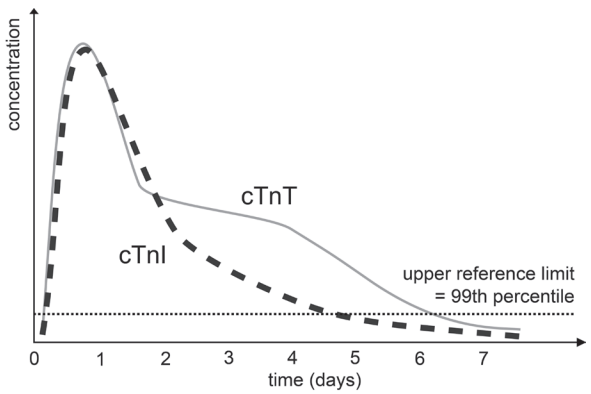

Figure 2. Schematic of the release kinetics of cardiac Troponin I (cTnI) and T (cTnT) after an acute myocardial infarction (occurring at t = 0). The dotted line represents the upper reference limit.

(77). This is no small feat as, contrary to the human genome which is more or less constant, the proteome is dynamic and changes constantly in response to the specific requirements of a cell. In addition, each distinct protein can be altered or modified in numerous ways, creating molecularly distinct variants of the same protein.

This can occur through RNA splicing, mutations, proteolysis, or other posttranslational modifications. The term "proteoform" was introduced to designate all of the different molecular forms of a protein encoded by a single gene (78).
While proteomics usually concerns the identification of huge amounts of proteins using techniques such as (2D) gel electrophoresis, Western blotting, and mass spectrometry, these techniques can also be used to learn more about the different proteoforms of cTnT. Specifically, we will use these techniques to identify different cTnT molecules during various stages of the cTnT lifespan.

The proteomics workflow
During the past two decades of proteomics research, numerous workflows have been created to effectively identify and quantify thousands of different proteins and protein modifications (77, 79-82). Although different methods of enrichment, labelling, digestion, fractionation, and detection are used in each workflow, the available workflows can be generalized into a basic scheme containing the essential elements of a proteomics experiment (**figure 3A**). The general workflow can be expanded upon in many ways according to the conditions of a specific experiment or research question. The different steps also need to be filled in as there are different enrichment strategies available, different enzymes to use during digestion, and different mass spectrometry instruments and settings.
For the majority of studies in this thesis a gel-based approach is chosen which mimics the detection mechanism of the clinical cTnT assay, based on the original experiments by Michielsen et al. (83). For this approach, cTnT is first enriched from human serum using an immunoprecipitation technique employing the M11.7 catcher antibody by Roche Diagnostics, coupled to magnetic beads with which individual cTnT molecules can be isolated from serum. Captured cTnT is eluted from the beads and its different molecular products are then subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), which is able to separate proteins based on their molecular size. After transferring the proteins to a nitrocellulose membrane, the different cTnT products are visualized with the Roche M7 detector antibody (Western blotting) and subsequently individually excised from the gel to be analysed with mass spectrometry (**figure 3B**).

Mass spectrometry
The identification of proteins using mass spectrometry is now considered to be the 'Gold standard' of protein identification and has replaced the Western blot in

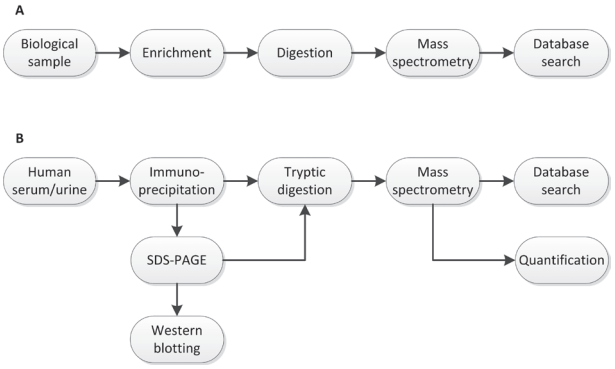

Figure 3. A. Generalized proteomics workflow containing the most basic steps essential to a proteomics experiment. **B.** Proteomics workflow as used in the studies presented in this thesis. The majority of experiments follow the "immunoprecipitation, SDS-PAGE, digestion"-route. Some experiments with urine samples skip the SDS-PAGE step and go straight to in-solution digestion after enrichment of cTnT.

terms of specificity, but not yet sensitivity (84, 85). In this thesis, the identification of proteins is done using the "bottom-up principle", where proteins in a sample are first digested into peptides using an enzyme, usually trypsin (86, 87). Trypsin is a highly specific enzyme which cleaves at the carboxyl-terminus of every Arginine and Lysine residue, except when followed by a Proline. This typically creates peptides of 6-12 amino acids in length; ideal for proteomic experiments.

The digests are then introduced into the mass spectrometer where the peptides are first ionized. Ionization is a critical step in mass spectrometry as uncharged particles are not affected by the electromagnetic fields inside the instrument. Two ionization techniques are commonly available to ionize peptides and proteins: Matrix-Assisted Laser Desorption Ionization (MALDI) (88) or Electrospray Ionization (ESI) (89). MALDI works by covering the sample in a crystallized matrix on a sample plate. A pulsed laser then irradiates the sample, vaporizing and (de)protonating the analytes within. In contrast, ESI is an online ionization technique that requires a steady flow of sample. It is therefore ideal to be combined with a chromatographic separation technique like ultra-high performance liquid chromatography (UHPLC). In UHPLC, a pressurized solvent containing the sample mixture passes through a column packed with adsorbent material such as C18. Analytes in the sample adhere to this material and can be eluted at different times based on their physical properties (like hydrophobicity in the case of C18) (90). Since the elution time, also called retention time, is different for different analytes, UHPLC can be used as a pre-separation step for mass spectrometry. Peptides that elute at a specific time are subjected to ESI, where a high voltage is applied to the sample flow causing aerosols to form. Evaporation of the solvent and electrostatic repulsion quickly reduces the size of the aerosols until only the charged species remain as gas-phase peptide ions. ESI can result in a wide range of charges applied to single compounds.

The Q Exactive hybrid-Orbitrap mass spectrometer by ThermoFisher Scientific (91), which is used throughout this study, utilizes ESI as its main source of ionization. Charged particles are guided using electromagnetic lenses towards the quadrupole mass filter which can rapidly select ions in a narrow m/z range. The selected ions are accumulated in the C-trap until a certain number of ions is acquired. Accumulated ions are then either sent directly to the Orbitrap mass analyser for the detection of the MS1 spectrum, or to the HCD-cell (higher-energy collisional dissociation) for fragmentation. **Figure 4** shows a schematic of the instrument.

In discovery (or "shotgun") proteomics, a "full scan" is acquired using a very wide isolation window (200 – 1500 m/z), allowing as much ions to be detected as possible. The most abundant precursor ions (i.e. the highest peaks) are then selected using a very narrow isolation window (typically 2 Da) and subjected to the HCD-cell for fragmentation. Ions that enter the HCD-cell will collide with nitrogen gas that is present in the cell at a pressure of 5 mbar (92). These collisions will result in the dissociation of the peptides (precursor ions) into different fragments (product ions) denoted a-, b-, and c-ions when counted from the N-terminus, and x-, y-, and z-ions when counted from the C-terminus of the peptide backbone (**figure 5**) (93). The amide-bond is the weakest link in the peptide structure and will therefore result in the highest number of fragments. Because the positive charge is usually retained on Arginine and Lysine residues, the y-ion series is usually the most abundant, followed by the b-ion series. After HCD fragmentation, the product ions are again accumulated in the C-trap and subsequently sent to the Orbitrap mass analyser for the acquisition of the MS2 (MS/MS, or product) spectrum.

The product ion spectra acquired with the mass spectrometer are then matched with theoretical spectra of in silico digested proteins present in a database. Using advanced search algorithms such as SEQUEST (94), Mascot (95), and others, these so-called peptide-spectral matches (PSMs) are given a score based on the agreeability of the match. However, due to random chance, it is possible that these matches are assigned incorrectly. It is therefore necessary to define a cut-off score based on the acceptable false discovery rate (FDR); the percentage of accepted PSMs that are incorrect (96). Statistical software such as Percolator (97) determines this cut-off by performing a "decoy" search of the acquired MS/MS spectra versus a dummy database (98, 99). All PSMs resulting from the decoy search are incorrect, giving an estimation of the number of incorrect PSMs in the real search. Typically, FDR thresholds of 1% are used, meaning that in these cases, 99% of the accepted PSMs are correct.

Targeted proteomics

While the previously-described shotgun proteomics approach allows for high-throughput and the identification of thousands of proteins simultaneously, it suffers from low reproducibility and sensitivity,

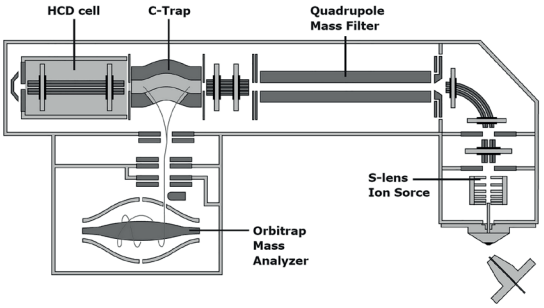

Figure 4. Schematic of the Q Exactive hybrid-Orbitrap mass spectrometer by Thermo Scientific (not to scale). Image adapted from www.planetorbitrap.com.

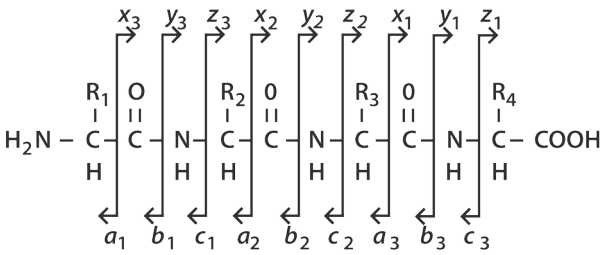

Figure 5. Product ion formation after collision of peptide ions with nitrogen. y-, b-, and a-ions are the most common product ions. c-, x-, and z-ions are encountered sporadically.

and inconsistent results when analysing similar samples (100). Targeted proteomics is an alternative approach to proteomics that tries to alleviate some of these drawbacks by selectively targeting ions of interest. In this approach, preselected ions are filtered by the quadrupole using a narrow isolation window. This narrow mass window, combined with the high-resolution and accurate mass determination (HR/AM) of the Orbitrap, allows for the systematic removal of background interferences and results in increased sensitivity, reproducibility, and even selectivity (82, 101). In addition, it is possible to increase the accumulation time of selected precursor ions in the C-trap to further increase the sensitivity of the method. Targeted proteomics is a promising new approach to mass spectrometry that changes the field from a discovery-driven approach to a hypothesis-driven approach and that is becoming more and more accessible to basic researchers in the field. No wonder that Nature Methods declared this technique to be "method of the year" in 2012 (102).

The Q Exactive instrument is capable of performing two types of targeted measurements: Selected Ion Monitoring (SIM) and Parallel Reaction Monitoring (PRM) (103). In PRM, the selected precursor ions are accumulated in the C-trap and subsequently fragmented in the HCD-cell. Only product ion spectra (MS2) are acquired in a PRM experiment, resulting in maximum selectivity. However, as the total signal of a precursor ion is being "diluted" into multiple product ions, the sensitivity will be increased when no product ion spectra are taken (104). In a SIM scan, accumulated precursor ions are directly measured in the Orbitrap at the MS1 level, resulting in reduced selectivity, but maximum sensitivity. Because of the low cTnT-concentration in our samples, the majority of MS experiments in this thesis are based on SIM scans. These scans are combined with a single, data-dependent, MS2 scan event. This allows for the identification of the peptide of interest and is used to circumvent the low selectivity of SIM. In the remainder of this thesis, this measurement strategy is called: "targeted-Selected Ion Monitoring with data-dependent tandem-MS" (t-SIM/dd-MS2).

Thesis outline

Cardiac troponin T (cTnT) is a highly specific and sensitive biomarker for the diagnosis of acute myocardial infarction (AMI) and is subjected to posttranslational modifications during both its physiological and clinical lifespans. In this thesis we will apply different proteomic approaches to unravel the nature of these modifications in different parts of the cTnT lifecycle. In **chapter 2**, we start our investigations by discussing the modifications that occur during the physiological lifespan of cTnT. We show that cTnT can be phosphorylated by protein kinase C (PKC) and that this phosphorylation pathway can be altered under pathological conditions possibly influencing cardiac output. We also briefly discuss the N-terminal degradation of cTnT and its effect on cardiac muscle contraction. In **chapter 3**, we have established a mouse ischemic cardiomyocyte cell model to study the release of cTnT from the heart to the blood circulation. This release marks the transition from the physiological to the clinical phase. **Chapter 4** focuses on the development of a targeted mass spectrometry assay able to differentiate different forms of degraded cTnT. This assay was extensively validated by spiking recombinant cTnT in a serum pool of healthy individuals. In **chapter 5**, we apply this targeted mass spectrometry assay to the serum of patients suffering from AMI and prove that cTnT is fragmented in these samples. Moreover, the amino acid sequence of these fragments was reconstructed. In **chapter 6**, we argue that the degradation of cTnT in the blood circulation of patients is in fact caused by a plethora of proteases, including the coagulation factor thrombin. **Chapter 7** shows that the same absolute amount of cTnT, added to the serum of different healthy individuals, can result in the determination of vastly different cTnT concentrations. Finally, **chapter 8** will discuss the end of the cTnT lifecycle and suggests that cTnT may, at least in some cases, leave the blood circulation via urine. A general discussion of the results obtained in this thesis and a direction of future research will be given in **chapter 9**.

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