Chapter 7

Discussion & future perspectives
Heart failure (HF) is the most prominent health challenge of the developed world, with a five year survival rate of less than 50% [1]. HF is defined as the complex end stage clinical syndrome that can result from numerous cardiac disorders, including myocardial infarction (MI), hypertension, cardiomyopathies, and valvular disease. Although, many breakthroughs have been made, the fundamental mechanisms responsible for the development and progression of HF have not yet been fully elucidated. In recent years it has been observed that cardiac injury in the adult heart leads to a switch in gene expression which to some extend resembles the expression pattern observed in the fetal heart. This process has been described as cardiac fetal reprogramming, and is defined as the suppression of adult and re-expression of fetal genes in the diseased myocardium. The exact reasons and mechanisms as to why the adult heart reverts back to a fetal-like expression pattern and the consequences here of remain unknown. With this thesis we provide a large body of evidence suggesting that a better understanding of cardiac fetal reprogramming can lead to novel therapeutic strategies for patients with HF. We describe the identification of OPLAH, a novel member of the cardiac fetal gene program, which possesses a cardio-protective effect by scavenging the oxidative stress inducing metabolite, 5-oxoproline. In turn, we demonstrate 5-oxoproline to be a putative novel biomarker for patients with HF.

Cardiac fetal reprogramming: a tool to exploit novel treatment targets for the failing heart

In chapter 2, we reviewed the current knowledge regarding cardiac fetal reprogramming in HF, by looking at the expression profiles during cardiac development and disease, with a particular focus on cardiac metabolism, contractile machinery, electrophysiology, and neurohormonal expression.

In terms of cardiac metabolism we describe how during development the heart reverts to a fetal pattern in which glycolysis primarily contributes to ATP production as opposed to fatty acid oxidation [2,3]. This switch in energy substrates has been suggested to result from a reduction in PPAR-α and PGC-1α levels due to rising levels of HIF-1α [4]. Once HIF-1α levels increase in the adult heart, expression of 6-phosphofructo-2-kinase (PFK2) increases, resulting in increased levels of fructose-2,6-biphosphate, thereby activating PFK1 and ultimately glycolysis [5].

Besides the switch in energy metabolism, cardiac fetal reprogramming also occurs in the contractile machinery. Maturation from a fetal to an adult heart involves a steady shift from compliant (fetal) to stiffer (adult) contractile proteins. As a result of cardiac disease, the adult heart undergoes a reversion to a more compliant fetal contractile machinery. This turnover has been highly studied in the sarcomere, where several components of the sarcomere revert to a more fetal like state upon cardiac injury.
Myosin heavy chain (MHC) and light chain (MLC) are part of the “molecular motor” of the sarcomere, and both have isoforms that are mainly expressed in the fetal and mature heart. Studies have demonstrated that an isoform switch takes place as a result from cardiac injury that resembles the fetal heart for both the MHC and the MLC (6–11). Similar isoform switches have been observed for actin (10–14), troponin (14–16) and titin (14,17–19).

The reversion to a more fetal-like state in response to cardiac injury has also been observed in the mechanisms regulating the electrophysiology of cardiomyocytes. Cardiomyocyte electrophysiology is in large part governed by the expression of ion channels, gap junctions, and the calcium homeostasis. It has been observed that the immature and mature heart differ from each other in terms of excitability, action potential properties, contractility, and relaxation (20). As a result the fetal heart expresses different genes involved in the generation and propagation of the action potential then the adult heart. Sodium channels (I_{Na}), potassium channels (I_{Ko}, I_{Kf}, I_{Ks}), and calcium channels (I_{Ca,L} and NCX) are expressed lower in the fetal heart then in the adult heart, on the other hand the I_{Ca,T} (CACNA1H) and I_{f} (HCN4) ion channels are significantly higher expressed in the fetal heart then the adults heart (20). Interestingly, in cardiac tissue of patients diagnosed with end stage HF, the major sodium (I_{Na}), potassium (I_{Ko}, I_{Kf}, I_{Ks}), and calcium (I_{Ca,L} and NCX) ion channels are significantly repressed, while I_{Ca,T} and I_{f} (HCN4) are up-regulated (21–23). These findings suggest that as a result of cardiac injury, the heart undergoes ion channel remodeling, resulting in an expression profile similar to that of the fetal heart. Likewise, it has been well established that during cardiac development in both rodent models and in the human setting, the expression of connexin 43, connexin 40, and connexin 45 are progressively increased as the heart matures (24–27). This developmental increase in the density of gap junctions in the heart correlates well with the developmental increase in conduction velocity. Upon cardiac damage in both rodent models and in the human setting, connexin 43 expression is not only drastically reduced (±50%), but the remaining connexin 43 gap junctions are also highly disorganized (27–29). This decrease in connexin 43 expression is also associated with an increase in connexin 40 expression (27–29).

Finally, cardiac fetal reprogramming is not only limited to metabolism, contractile machinery, and electrophysiological, but also occurs in the expression of cardiac neurohormones. Specifically fetal reprogramming has been observed in the expression of atrial and brain natriuretic peptides (ANP and BNP, respectively). Both ANP and BNP are highly expressed in the fetal heart, and as the heart matures these levels drop rapidly. Following cardiac injury, these two neurohormones are re-expressed. In recent years it has been well established that the re-expression of
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both ANP and BNP has a cardioprotective effect in the failing myocardium.

Combined these observations all suggest that cardiac fetal reprogramming is an integral part of the pathophysiology of HF. Although there is plethora of evidence regarding cardiac fetal reprogramming and its importance during HF, there still remains much to be uncovered regarding this process. In this thesis I describe how understanding the process of cardiac fetal reprogramming can lead to novel therapeutic strategies for HF patients.

Identification of OPLAH as a novel member of the cardiac fetal gene program, and its cardio-protective effect in myocardial dysfunction

In chapter 3 we sought to identify new members of the cardiac fetal gene program and investigate whether these genes could serve as therapeutic targets in the human setting. By means of RNA sequencing analysis on different stages of murine cardiac development and disease, we identified 68 genes which behaved like cardiac fetal genes (demonstrating inverse expression during cardiac disease as compared to cardiac development). Of these 68 putative cardiac fetal genes, 39 had already been described in the literature as associated with cardiac disease or development (including Ryr2, Cacna2d1, Fstl1 and Bambi) (30–33). The remaining 29 genes had been associated with neither cardiac development nor cardiac disease to date, and were considered novel genes associated with the cardiac fetal-like gene program. To evaluate whether these 29 putative cardiac fetal-like genes were also relevant in humans, we screened the expression of these genes across an adult human organ panel using qRT-PCR. ANXA11, HADH, CD300LG, and OPLAH were predominantly expressed in the human heart. Of these four genes, OPLAH, was found to be the more cardiac specific and as such we further explored the role of OPLAH in the heart.

OPLAH is a gene which encodes for 5-oxoprolinase an enzyme involved in the γ-Glutamyl cycle, where it specifically converts 5-oxoproline, a degradation product of glutathione (GSH), into glutamate (34,35). We identify OPLAH as a novel cardiac gene involved in HF, which is at least in part regulated by the PGC-1α/ERRα axis. Both PGC-1α and ERRα are key transcriptional regulators of antioxidant protection genes (36). It has been well established that PGC-1α regulates ERRα expression, and that the expression of PGC-1α is induced in cardiac development and repressed in HF (36,37). Our data support the interaction between PGC-1α and ERRα by demonstrating that direct inhibition of ERRα activity results in an increase in PGC-1α and ERRα mRNA, suggesting a compensatory mechanism. Furthermore, the decreased activity of ERRα was shown to reduce OPLAH expression and enhance oxidative stress. These observations are in line with a recent study, which demonstrated by microarray analysis on RNA isolated from ERRα knockout mouse
hearts that these mice had significantly increased expression of PGC-1α and reduced OPLAH (38).

In chapter 3 we also investigated the consequence of OPLAH depletion in HF resulting in an increase in oxidative stress and 5-oxoproline. Furthermore, exogenous administration of 5-oxoproline to cardiomyocytes also led to increased oxidative stress. This finding is supported by a previous study that identified 5-oxoproline as an inducer of oxidative stress in brain tissue (39,40). We propose that HF leads to the reduction of PGC-1α, which, as a consequence, results in a decrease in ERRα and antioxidant protection genes, including OPLAH. Due to reduced OPLAH expression, 5-oxoproline cannot be processed into glutamate, and the excessive accumulation of 5-oxoproline leads to increases in oxidative stress, adding further insult to the progression of the disease. By exposing mice with cardiac-specific OPLAH overexpression to cardiac injury, we demonstrate that these mice have less oxidative stress, lower 5-oxoproline, and reduced fibrosis, resulting in improved cardiac function. Thus, we posit that OPLAH is a potential target for therapeutic intervention in HF.

To further stress the involvement of OPLAH and 5-oxoproline in HF, we measured 5-oxoproline in plasma of both experimental and clinical HF. In rats with pressure overload–induced HF (REN2) where LV tissue 5-oxoproline was ~20-fold higher than in control rats, circulating 5-oxoproline was also found to be about six fold higher. To determine whether these findings could be extrapolated to the human setting, we measured 5-oxoproline in the plasma of healthy controls (n = 10) and patients with acute HF (n = 10) (24). Plasma 5-oxoproline was increased about four fold in acute HF patients compared to healthy controls. To assess the potential of 5-oxoproline to serve as a circulating biomarker in clinical HF, we tested the prognostic potential of 5-oxoproline in a cohort of 535 patients who had been hospitalized for acute HF. Interestingly, we found that higher 5-oxoproline was associated with a worse outcome. These findings suggest that circulating 5-oxoproline, the substrate of OPLAH, can serve as a potential biomarker in patients with HF, further stressing the involvement of OPLAH and 5-oxoproline in HF.

**OPLAH ablation leads to accumulation of 5-oxoproline, oxidative stress, fibrosis and elevated fillings pressures in a murine model for heart failure with a preserved ejection fraction.**

In chapter 4, we sought to further characterize the role of OPLAH in HF. In chapter 3 we found OPLAH to possess a cardio-protective effect in an over-expression mouse model (41). Furthermore, in our in vitro work we demonstrated that a reduction in OPLAH resulted in increased susceptibility towards oxidative stress (41). To further address this observation, in chapter 4 we describe the development of an Oplah full
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body knock-out (KO) mouse and the effects ischemia/reperfusion (IR) injury has on these mice.

OPLAH ablation in mice was observed to result in increased 5-oxoproline, oxidative stress, atrial enlargement, fibrosis, ventricular filling pressures, and impaired LV relaxation coupled to a preserved LV ejection fraction. Interestingly, at baseline these mice did not only develop a cardiac phenotype, but we also observed an increase in renal fibrosis, suggesting the increase in 5-oxoproline was resulting in renal damage. Interestingly, when challenged, these mice were found to be more susceptible to cardiac damage and sudden death, following cardiac IR injury. Furthermore, genetic disruption of Oplah not only led to an increase in cardiac damage, but we also observed that the Oplah KO mice developed proteinuria following IR injury. Combined these findings suggested that OPLAH ablation in mice resulted in a phenotype reminiscent of patients with HF with preserved ejection fraction (HFpEF). This in its self is novel since to date there are no strong animal models to study HFpEF. Thus utilizing these mice my serve to better understand this disease and uncover novel therapeutic strategies to treat the disease.

As described in chapter 3, we identified that 5-oxoproline was not only elevated in the myocardium but also in the plasma of animals with HF. In acute HF, circulating 5-oxoproline was independently associated with patient outcome and associated with known markers for cardiac remodeling, stretch, and oxidative stress, but not with markers for inflammation (41). To further explore the potential of 5-oxoproline as a biomarker in HF, and in particular with regards towards its involvement in HFpEF, in chapter 4 we measured this metabolite in a small cohort of patients with HFpEF. Similar to our observations in chapter 3 where 5-oxoproline levels were increased in patients with acute HF when compared to healthy controls, we found that plasma levels of 5-oxoproline were also higher in HFpEF patients then healthy controls. Furthermore, higher levels of circulating 5-oxoproline were found to independently associate with more concentric remodeling, a hallmark of HFpEF.

Although our proposed murine model for HFpEF mimics HFpEF in the human setting, there remain several limitations to this model. The main limitation is that in this model HFpEF is developed as a result of direct genetic manipulation. Thus, it is uncertain whether the pathophysiological pathway, implicating OPLAH and 5-oxoproline, is also involved in the onset of HFpEF in humans. Rather one could also speculate that the effects we observe in the Oplah KO mice are a result of the severe oxidative stress, due to accumulation of the oxidative stress inducing agent 5-oxoproline. Additionally, circulating 5-oxoproline was measured in a very specific cohort of HFpEF patients with pulmonary hypertension, and therefore it is
uncertain to which extent findings of this patient cohort can be extrapolated to other patients with HFpEF. Furthermore, the sample size was rather small, inhibiting us from performing more extensive analyses.

**LC-MS analysis of key components of the γ-Glutamyl cycle in tissues and body fluids from mice with Myocardial Infarction**

In chapter 3 and chapter 4 we utilized a LC-MS method that enabled us to measure 5-oxoproline and glutamate. However to obtain a better understanding of the involvement of the γ-glutamyl cycle in heart failure, in chapter 5, we sought out to develop a LC-MS method for the quantification of 5-oxoproline, glutamate, GSH and GSSG (oxidized GSH), key components of the γ-glutamyl cycle, in biological samples. The method we developed and validated, accurately and reliably quantified 5-oxoproline, glutamate, and GSH. The levels of GSSG were quantifiable in murine tissues, however this was not the case for plasma and urine of these animals. The fact that GSSG was not detectable in the plasma and urine samples suggest that within these samples GSSG is either unstable or the GSSG levels within the samples falls out of the lower limits of the detection of the method.

Utilizing the developed methodology, we assessed the effects on the γ-glutamyl cycle following the induction of HF in mice. Specifically in the heart we found increases in 5-oxoproline together with a decrease in the GSH/GSSG ratio in mice exposed to HF, further strengthening the notion that 5-oxoproline is an oxidative stress inducing agent (39–41). In addition to being elevated in the heart, 5-oxoproline levels were found to be increased in the kidney, liver, plasma and urine of all mice exposed to HF. However, the GSH/GSSG ratios in the kidney and liver of these animals remained stable, suggesting that these tissues have a higher buffering capacity for oxidative stress. Interestingly, we found 5-oxoproline levels in urine to be elevated following cardiac injury, suggesting that, like plasma, urine 5-oxoproline levels could serve as a possible HF biomarker.

**Treating oxidative stress in heart failure: past, present, and future**

OPLAH is a member of the γ-Glutamyl cycle, and following the observations made in this thesis, we were interested in further characterizing this cycle in HF and uncover whether other members of this cycle could also serve as possible therapeutic targets for patients with HF. To this end, in chapter 6 we reviewed the current knowledge regarding the γ-Glutamyl cycle and its association to oxidative stress and HF.

The γ-Glutamyl cycle is responsible for the GSH metabolism. GSH is synthesized from glutamate, cysteine, and glycine by γ-glutamylcysteine synthetase (GCL) and glutathione synthetase (GS) (42). Upon synthesis, GSH can be utilized internally by the cell, or be exported to the extra-cellular matrix. The primary means of recovering
the exported GSH is by a scavenging pathway, involving γ-Glutamyltransferase (GGT), γ-glutamylcyclotransferase (GGCT), OPLAH, and dipeptidase (42).

GSH is the major source of non-enzymatic antioxidants, but also participates in biosynthetic pathways, signaling processes, detoxification, and storage and transport of key metabolites (35). Its function as an antioxidant is regulated by glutathione peroxidase (GPx) and glutathione reductase (GR). GPx utilizes two GSH molecules as electron donors in the reduction of hydrogen peroxide to water, producing glutathione disulfide (GSSG) in the process (43). Once GPx oxidizes GSH to GSSG, GSH reductase (GR) can reduce GSSG back to GSH at the expense of NADPH. The ratio of GSH to GSSG largely determines the intracellular redox potential. When oxidative stress overcomes the cell's ability to reduce GSSG to GSH, GSSG is actively exported out of the cell to prevent a major shift in the redox equilibrium (44). Therefore, severe oxidative stress depletes the intracellular GSH pool (45).

Several studies focused on the γ-Glutamyl cycle have uncovered that certain enzymes involved in this cycle have cardio-protective properties, including GCL, GPX, and OPLAH. However, clinical strategies for targeting these steps have not yet been explored, which is in large part due to the lack of drugs or small molecules that specifically target these enzymes. The research focused on dissecting the involvement of the γ-Glutamyl cycle and GSH in HF has not only resulted in the identification of novel therapeutic targets for this disease, but has also lead to the characterization of several novel oxidative stress-associated HF biomarkers. Plasma GSH concentrations have been shown to be highly associated with cardiac disease severity (46–48). 5-oxoproline, a degradation product of GSH and an oxidative stress-inducing metabolite, has recently also been shown to associate with outcome in patients with HF. Likewise, serum GGT levels were found to be significantly associated with CVDs risk. Finally, both GR and GPx-1 activity, measured in patient plasma, were shown to be predictors for cardiovascular risk. These findings further address the importance GSH and the γ-Glutamyl cycle have in the development and progression of HF.

**Future Perspectives**

Given the high morbidity and mortality rate in heart failure, the identification of new pathways and therapeutic targets is crucial. These efforts may eventually lead to the discovery of novel therapies for HF. To this end in the work described in this thesis we tried to uncover novel therapeutic candidates by looking at the process of fetal reprogramming, a process where the gene expression profile of the diseased heart resembles that of the developing heart, leading to the discovery of OPLAH and 5-oxoproline.
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Besides the work covered in this thesis, limited information is available in the current scientific literature regarding OPLAH in particular in the HF setting. Furthermore, to date there are no known compounds which have the capacity to specifically increase the expression or activity of OPLAH. Thus the development of an innovative drug screening tool for OPLAH expression or activity may lead to the identification of novel therapeutic strategies and improved prognosis for patients with HF. The most applicable technique to increase the expression of OPLAH in patient hearts would involve some form of genetic manipulation, such as gene therapy. However, this technique carries a high risk to it, and its safety and effectiveness remains unproven (49). One could also induce OPLAH expression by targeting ERRα, however this transcription factor targets a large number of other genes (including genes involved in energy metabolism, mitochondrial oxidative metabolism, mitochondrial biogenesis, lipid metabolism and carbohydrate metabolism) and it is uncertain what the safety, effectiveness, and whether this would lead to any beneficial effects. Thus, the most clinically viable therapeutic option would be to identify a highly specific and selective compound capable of increasing the endogenous OPLAH activity.

Another way one could envision the work covered in this thesis to translate into the clinic would be in utilizing 5-oxoproline as a diagnostic and/or prognostic biomarker for patient with HF. Although it is still not certain whether the 5-oxoproline present in plasma is directly linked to the reduction of OPLAH in the myocardium, the association we show throughout this thesis is that plasma 5-oxoproline is clearly elevated in HF patients. In patients with acute HF plasma 5-oxoproline is highly associated with patient outcome. Interestingly, since 5-oxoproline is associated with oxidative stress, and a reduction in OPLAH and an accumulation of 5-oxoproline lead to the development of a HFpEF-like phenotype in mice, suggests a possible link to HFpEF. When measured in a cohort of HFpEF 5-oxoproline was found to be elevated and associate with concentric remodeling. This observation suggest that 5-oxoproline is also involved in the onset of clinical HFpEF. However, it is still not certain whether 5-oxoproline is a good biomarker to distinguish between HF patients with HFpEF and patients with reduced ejection fraction (HFrEF). To fully characterize 5-oxoproline as a HF biomarker it would be of interest to measure the plasma 5-oxoproline levels in a large cohort of HF patients, including both HFpEF and HFrEF patients. Finding 5-oxoproline to be specific for patients with HFpEF, would be a major breakthrough since to date there are no available biomarkers that can specifically distinguish HFpEF from HFrEF. Furthermore we also demonstrate that 5-oxoproline levels in the urine of mice exposed to HF is also elevated. This is an interesting observation and could lead to a novel family of HF biomarkers which could be measured in the urine of HF patients, facilitating the ease of measurement and diagnosis. Thus, although the work in this thesis describes a novel putative
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HF biomarker, much remains unknown about the potential of 5-oxoproline as a diagnostic or prognostic marker in cardiovascular diseases.

Concluding remarks

With this thesis we aimed to provide a better understanding of fetal reprogramming in HF and how further unraveling this process may lead to novel therapeutic strategies that can benefit HF patients. To this end our work-flow consisted of four distinct steps. Firstly, we performed an RNA sequencing analysis on cardiac development and disease to help identify possible members of the cardiac fetal gene program. Secondly, putative candidate genes were screened in both in vitro and in vivo disease models to identify their importance in cardiac disease. Thirdly, we developed over-expression and knock-out murine models of our top candidate gene (OPLAH) to help unravel its role in myocardial dysfunction. Finally, by studying the pathophysiology of OPLAH we uncovered that its substrate, 5-oxoproline, could function as a possible novel biomarker in HF.

In this highly translational study, we went from gene discovery by means of RNA sequencing to in vitro and in vivo HF disease models, and finally transitioning into the clinical setting with the identification of a novel HF biomarker. Thus, this thesis offers a novel pathophysiological avenue in HF, that if further explored, could aid in the development of new therapeutic options for HF patients.
References

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