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Physiological Genomics of Heart Failure: From Technology to Physiology

Thesis for the degree philosophiae doctor

Trondheim, May 2007

Norwegian University of Science and Technology
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Funksjonell genomforskning og hjertesvikt:
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Hovedmålet for prosjektet var å identifisere molekylære mekanismer assosiert med økning av hjertestørrelse (hypertrofi) som følge av sykdom. Dette ble oppnådd ved blant annet å utvikle, etablere og benytte DNA mikromatriseteknologi og tilhørende dataanalysemetoder. Mikromatriseanalyser gjør det i prinsippet mulig å måle mengden mRNA (genutrykk) for alle genene i et genom (en organismes totale arvemasse) samtidig, i ett enkelt forsøk.

Hjerteprøver fra pasienter med hjertesvikt på grunn av koronar hjerte sykdom (blokkering av kransarterie som blant annet gir hjerteinfarkt) og sykdom i selve hjertemuskelen (kardiomyopati) ble undersøkt. Mikromatriseresultatene viste sykdomsspesifikke mønster, i hovedsak knyttet til stoffskifteprosesser, nedbryting og regulering av signalmolekyler. Det ble videre utviklet klassifikatorer (et sett med regler), basert på genuttrykks data fra mikromatriseanalysene. Klassifikatorene ble så brukt for å kunne forutsi om en ”ukjent” prøve kom fra en pasient med koronar hjerte sykdom eller fra en pasient med kardiomyopati. Disse forsøkene viser at i fremtiden vil slike metoder og teknologi muligens kunne bli brukt ved diagnostikk av hjertesykdom. I eksperimentelle rottemodeller, ble det så identifisert hittil ukjente og allerede kjente molekylære mekanismer assosiert med hjertehypertrofi indusert ved trening og hypertrofi indusert ved koronar hjertesykdom. Resultatene viste blant annet at ved sykdomsindusert hypertrofi blir gener assosiert med fettsyrestoffskiftet i hovedsak nedregulert. Dette skjer imidlertid ikke ved treningsindusert hypertrofi, noe som ser ut til å være en viktig forskjell mellom disse typene hypertrofirespons. Treningsindusert hypertrofi ble videre assosiert med mindre endringer på genuttrykksnivå, enn ved hva som ble observert ved koronar hjertesykdom. Dette indikerer at regulering på andre nivåer enn transkripsjon og genregulering, for eksempel fosforylering på protein nivå, kan være en viktig faktor ved treningsindusert hypertrofi. En av delstudiene viste at H^+/K^+ ATPase (protonpumpe) er uttrykt og regulert på mRNA og protein nivå i både hjerteceller og hjertevev. Studier av levende hjerteceller indikerte videre at H^+/K^+ ATPase kan stå for opp mot 25% av kaliumopptaket over cellemembranen. Dette indikerer at proton pumpen kan være en viktig mekanisme for pH- og kaliumregulering.

En viktig del av prosjektet var utvikling og bruk av programmet *GeneTools*, et ”alt i ett annoteringsverktøy”. *GeneTools* består av informasjon fra eksterne databaser (blant annet Entrez, Gene Ontology og SwissProt) samt at brukeren kan legge til og lagre egen definert informasjon. Videre inneholder *GeneTools* verktøyet *e*GOn, som tilbyr statistiske tester for å identifisere over/underrepresentasjon av sett/klasser med gener basert på informasjon om deres biologiske funksjon. Programmet er allerede blitt meget populært (>2000 brukere fra >50 land) på grunn av sin funksjonalitet og enkelt brukergrensesnitt.

Contents

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Finally and most of all, I want to thank my family for their support during all these years. Without your encouragement, this had not been possible.

PS! This thesis has been written by listening to, and probably influenced by music. The following pieces have been important to me and can be recommended as listening, especially if you intend to read this thesis.

1. GP/Grievous Angel, Gram Parsons (1973/1974)
2. Kicking Television – Live in Chicago, Wilco (2005)
3. Feast of Wire, Calexico (2003)
4. White Blood Cells, The With Stripes (2001)
5. Grace, Jeff Buckley (1994)
6. Theo Buhara Presents: The Tussler – Original Motion Picture Soundtrack, The International Tussler Society (1994)
7. Olsen´s Lot, Midnight Choir (1996)
8. Demon Box, Motorpsycho (1993)
9. Harvest, Neil Young (1972)
10. Nixon, Lambchop (2000)

Trondheim, January 2007

Vidar Beisvåg

Preface

This thesis for the Doctoral Degree PhD in Molecular Medicine is based on four studies/papers listed below, referred to by roman numerals in the text. The work was carried out at the Norwegian University of Science and Technology, at the Department of Circulation and Medical Imaging and in collaboration with the Department of Cancer Research and Molecular Medicine.

Paper I

Vidar Beisvag, Geir Falck, Jan P. Loennechen, Gunnar Qvigstad, Per Jynge, Tor Skomedal, Jan B. Osnes, Arne K. Sandvik and Øyvind Ellingsen. Identification and regulation of the gastric H^+/K^+-ATPase in the rat heart. Acta Physiol Scand. 2003 Nov;179(3):251-62.

Paper II

Vidar Beisvag, Per Kristian Lehre, Herman Midelfart, Halfdan Aass, Odd Geiran, Arne K. Sandvik, Astrid Lægreid, Jan Komorowski and Øyvind Ellingsen. Aetiology-specific patterns in end-stage heart failure patients identified by functional annotation and classification of microarray data. Eur J Heart Fail. 2006 Jun;8(4):381-389.

Paper III

Vidar Beisvag, Frode K. R. Jünge, Hallgeir Bergum, Lars Jølsum, Clara-Cecilie Günther, Stian Lydersen, Heri Ramampiaro, Mette Langaas, Arne K. Sandvik and Astrid Lægreid. GeneTools – Application for Genomic Functional Annotation and Statistical Analysis. BMC Bioinformatics. 2006 Oct 24;7(1):470.

Paper IV

Vidar Beisvag, Ole J. Kemi, Mette Langaas, Ingerid Arbo, Jan P. Loennechen, Ulrik Wisløff, Arne K. Sandvik, and Øyvind Ellingsen. Serial gene expression and functional annotation analysis of pathological and physiological hypertrophy in the rat heart. Submitted January 2007.

Summary

Genome wide gene expression in cardiac disease is incompletely characterized. The main purpose of this project was to increase insight into molecular mechanisms of myocardial hypertrophy and heart failure in experimental models and human disease. We aimed to establish and use microarray technology and bioinformatics tools to obtain these results. Finally, we sought to relate gene/protein expression to function in vitro, by functional studies in isolated cardiac myocytes.

Microarray technology and methods of data analysis were established which enabled detection of differentially expressed genes. Combining gene expression data and functional annotations yielded a biologically meaningful analysis which identified potentially important molecular mechanisms of end-stage heart disease and physiological hypertrophy. Gene expression classifiers were developed to distinguish between myocardial samples from end-stage heart failure, originating from either coronary artery disease or dilated cardiomypathy. Gene-class testing analysis indicated aetiology-specific patterns in coronary artery disease and dilated cardiomypathy, primarily related to genes involved in catabolism and regulation of protein kinase activity. Serial cardiac-specific gene expression was studied during the development of hypertrophy in congestive heart failure and exercise training. Our results suggest that one of the main molecular differences could be down-regulation of fatty acid metabolism genes, which was observed in pathological hypertrophy but not in exercise-induced hypertrophy. Congestive heart failure was associated with more comprehensive changes in gene expression than exercise training. This indicates that post-transcriptional and post-translational regulation may be important in physiological hypertrophy. All gene/protein annotations and gene-class analyses were generated by *GeneTools*, a program that was developed in our group during the project as an "all in one" annotation tool.

In isolated rat ventricular cardiomyocytes, we showed that H^+/K^+-adenosine triphosphatase was expressed and regulated both at the transcript and protein level. Functional in vitro studies indicated that the H^+/K^+-ATPase may account for up to about 25% of the K^+-uptake across the ventricular sarcolemma.

Abbreviations

BP	Biological Process
CAD	Coronary Artery Disease
CC	Cellular Component
cDNA	Complementary DNA
CHF	Congestive Heart Failure
Cy	Cyanidin
DAG	Direct Acyclic Graph
DCM	Dilated Cardiomyopathy
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EST	Expressed Sequence Tag
FA	Fatty Acids
GO	Gene Ontology
HF	Heart Failure
LV	Left Ventricle
MF	Molecular Function
MI	Myocardial Infarction
MIAME	Minimum Information About Microarray Experiments
mRNA	Messenger RNA
PCR	Polymerase Chain Reaction
QC	Quality Control
RNA	Ribonucleic acid
RV	Right Ventricle
rt-PCR	Reverse Transcriptase PCR
RT-PCR	Real Time PCR
SNP	Single Nucleotide Polymorphism

1. Introduction

1.1 Cardiovascular disease

Heart failure is a common end stage of cardiovascular disease and a leading cause of death worldwide [1]. However, in the recent years the "omics" technologies have resulted in new knowledge in cellular and molecular biology and have improved the understanding of the mechanisms of the disease and the possibility to design highly specific efficient drugs and other treatment strategies. In this way detailed knowledge of the basic mechanisms of heart failure has been and will be of vital importance and an important field of intensive research.

1.1.1 Heart failure pathophysiology

When a person is diagnosed with heart failure (HF), it does not mean the heart has stopped working, but rather that it is not working as efficiently as it should [2]. HF may occur suddenly, or it may develop gradually. When heart function deteriorates over years, one or more conditions may exist. The strength of muscle contractions is reduced, and the ability of the heart chambers to fill with blood may be limited by mechanical problems, resulting in less blood to pump out to tissues in the body. Conversely, the pumping chambers may enlarge and fill with too much blood when the heart muscle is not strong enough to pump out all the blood it receives [3]. In terms of histology, four features define the failing heart: myocyte hypertrophy, fibrosis, "slippage" of the previously orderly aligned myocytes which presumably leads to inefficient contraction, and apoptosis of myocytes. Together these processes are termed "remodeling" [4].

1.1.2 Causes

Several different issues can cause HF. The main cause is coronary artery heart disease (CAD) causing insufficient blood supply to the myocardium. CAD is usually caused by atherosclerosis, which is associated with the aggregation of lipids or plaque on the walls of the arteries. Then the heart's ability to perform decreases, because ischemia results in the delivery of less oxygen and nutrients to the heart muscle [5].

Cardiomyopathies are primary or secondary disorders of cardiac muscle associated with abnormal cardiac wall thickness, chamber size, contraction, relaxation, conduction, and rhythm. They are a major cause of morbidity and mortality at all ages and, like acquired forms of cardiovascular disease, often result in HF. There are three major types of cardiomyopathy,

dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), and restrictive cardiomyopathy (RCM) [6]. Causes of cardiomyopathy include infection, alcohol abuse, and cocaine abuse. However, cardiomyopathies are frequently hereditary and therefore subject to genetic studies. DCM can e.g. be caused by mutations at 25 chromosome loci where genes encoding contractile, cytoskeletal, and calcium regulatory proteins have been identified [7].
Long-standing high blood pressure (hypertension) is another common cause of HF and results in an increased heart muscle mass, especially of the left ventricle. In this way, left ventricular hypertrophy (LVH) is the most potent predictor of adverse cardiovascular outcomes in the hypertensive population, and an independent risk factor for coronary heart disease, sudden death, heart failure and stroke [8].

1.1.3 Treatment

Whenever possible, the best treatment of HF is one of prevention. This includes diagnosing and treating high blood pressure and attempting to prevent atherosclerosis [9,10]. A prudent diet, regular exercise, and weight control are also important [11]. When a patient is diagnosed as having HF, the first treatment is often restriction of dietary sodium. Diuretics help the kidneys to get rid of excess water and sodium, thereby reducing blood volume and working load of the heart [10]. Drugs for the treatment of HF include vasodilators, which cause the peripheral arteries to dilate. Standard vasodilators used for HF are the angiotensin-converting enzyme (ACE) inhibitors. ACE inhibitors block the production of angiotensin II (ANG II), a potent constrictor of blood vessels. Several landmark studies have demonstrated the effectiveness of long term treatment with ACE inhibitors in reducing the risk of death [12-14]. Other drugs used in the treatment of HF include beta blockers, which slow the heart. In the 1970s and 1980s beta blockers were commonly used on patients with HF, but mixed effects were shown. However, studies in the 1990s and later showed reduce risk of death with use of β-receptor antagonists, and combined treatment with ACE-inhibitors reduces mortality even more [15,16]. In addition to prevention and drugs, sometimes surgery proves effective. For example when HF is due to valvular disease, surgical implantation of an artificial heart valve or valve repair may relieve the problem [17]. Coronary artery bypass graft surgery [18] and coronary catheterization using balloon dilatation [19] are among the therapeutic techniques used to prevent and treat HF caused by blocked arteries. Heart transplants are a last resort in treating severe HF caused by diseased heart muscle. However, the cost of the operation and the shortage of donor organs make it impractical except as a last resort [20].

A new treatment of HF in the future might be cardiac stem cell therapy, which recently has raised many hopes. However, neither the ideal source and type of stem cell nor the critical cell number and mode of application have been defined so far [21].

1.1.4 Cardiac hypertrophy

Cardiac hypertrophy is an adaptive physiological response to increases in blood pressure that preserves myocardial wall stress, chamber size and contractile function. Despite these initial advantages, cardiac hypertrophy is also an independent risk factor for cardiovascular disease and, if left untreated, it frequently progresses to HF. In broad terms, there are three types of cardiac hypertrophy: normal growth, growth induced by physical conditioning (physiological hypertrophy), and growth induced by pathologic stimuli (pathological hypertrophy).

1.1.4.1 Relation of cardiac hypertrophy and heart failure

Clinically, the term "pathological" hypertrophy is used and referred to as an abnormal increase in cardiac mass, usually by an increase in the size of cardiac myocytes and an increase in the number of the fibroblasts and other cells. Precisely defined, hypertrophy exclusively refers to an increase in the volume of cardiac muscle cells.

Pathologic hypertrophy of cardiac myocytes is often a precursor of HF, and therefore research on the molecular pathways leading to hypertrophy can be considered as research into the initial step of HF [22,23]. It is also important to note that the molecular composition of the failing heart is relatively uniform and largely independent of the initiating injury or disease, as also described in Study II and IV.

The two major pathological stimuli for hypertrophy are mechanical stress and neural/humoral factors, which activate intracellular signaling pathways resulting in altered gene expression and protein synthesis, leading to an enlarged heart [24]. It has long been thought that "pathological hypertrophy" with fibrosis, occurring in hypertension and HF, is a useful adaptation of the heart to increased load, by analogy to the situation in athletes, who have "physiological hypertrophy" without fibrosis. This view has recently been changed and today it has been postulated that it is not hypertrophy per se that is detrimental but rather the balance of different signal pathways [25]. However, it remains true that pathological hypertrophy in humans is a frequent precursor to HF [26].

1.1.4.2 Physiological hypertrophy induced by exercise

Chronic exercise training can cause cardiac hypertrophy and is commonly referred to as "the athlete's heart" [27]. The athlete's heart is a physiological cardiac hypertrophy that is characterized by increases in left ventricle (LV) chamber size, wall thickness, and mass. Because the ratio of LV wall thickness to radius is unchanged, the athlete's heart is classified as eccentric LV hypertrophy. These adaptations can enhance cardiac function (e.g. LV diastolic filling) in the resting condition and help meet the increased cardiac demands during exercise [28]. On the other hand, pathologic cardiac hypertrophy is characterized by predominantly increased LV wall thickness with unchanged LV chamber size, which is known to be a precursor of heart failure [22,23]. Moreover, the athlete's heart does not result in dysfunction or heart failure, but is rather associated with sustained or improved contractile function. Since both athlete's heart and pathologic hypertrophy associate with cardiac growth, there may be similarities in the molecular mechanisms underlying pathologic LV hypertrophy and exercise training-induced physiological LV hypertrophy [29]. However, research on the molecular mechanism behind athlete's heart has so far been limited, but it is believed that the number and extent of such studies will increase in the near future, and the results may be important in understanding the differences in pathologic and physiological hypertrophy that can be important in treatment of heart disease.

1.1.5 Molecular mechanism in heart failure

The understanding of cardiovascular disease has evolved through the years by extensive studies emphasizing the identification of molecular and physiological mechanisms involved in normal and disease states. Major discoveries have been made along the way, e.g. it has long been known that HF is characterized by activation of the renin-angiotensin-aldosterone system (RAAS), catecholamine secretion and elevated cytokines in blood. This neuronal endocrine activation leads to progressive fluid retention as well as to increased peripheral vascular resistance. Most of the current standard treatment for HF (beta blockers and inhibitors of RAAS or of ANG II receptors) is based on this paradigm [30]. However, the majority of the work to find and explore the molecular mechanisms of HF has focused on specific genes or pathways rather than integrative approaches.

The microarray technology dramatically accelerated the speed of discoveries by giving us the ability to simultaneously study thousands of genes in a single experiment. Novel molecular mechanisms have been identified, known pathways are seen under new light, disease

subgroups begin to emerge, and the effects of various drugs are molecularly dissected. Many of the proteins of hypertrophied cardiac myocytes show quantitative alterations that are proportional to the increase in size of the cell. This is a result of an increase in translation efficiency [31]. In addition, a significant number of the ~10 000 genes expressed in cardiac myocytes are either newly expressed or show changes out of proportion with the degree of hypertrophy [32]. These expressed genes, related to multiple biological processes and pathways are responsible for transducing mechanical and hormonal stimuli in the process of HF. However, common for many of the microarray studies of HF, e.g. those reviewed by Kittelson et al. [33], is that a significant amount of the regulated genes mainly belong to functional categories of cell growth and maintenance, cytoskeleton/sarcomere, metabolism and signal transduction, which correlates with what we found in Study II and IV.

1.1.5.1 Metabolism

The metabolism of the failing heart is altered. Under normal circumstances, fatty acids are the principal energy source of the heart, but in pathological hypertrophy, metabolism is switched to glucose utilization [34]. This can also be clearly seen from the results of our Study IV. Acetyl-CoA derived from FA and glucose oxidation is further oxidized in the tricarboxylic acid cycle (TCA) to generate NADH and $FADH_2$, which enter the electron transport/oxidative phosphorylation pathway and drive ATP synthesis. Genes encoding enzymes involved at multiple steps of these metabolic pathways (i.e. uptake, esterification, mitochondrial transport, and oxidation) are regulated by the Peroxisome proliferator-activated receptor gamma (PPARγ) coactivator-1 (PGC-1α) with its nuclear receptor partners, including Peroxisome proliferator-activated receptors (PPARs) and Estrogen-related receptors (ERRs). It has been shown that PPARs also function as nuclear receptors for lipids and other metabolic substrates, and that they play a prominent role in this process [35]. Glucose uptake/oxidation and electron transport/oxidation phosphorylation pathways are also regulated by PGC-1α via other transcription factors, such as MEF-2 and NRF-1. It is therefore hypothesized that one of the key mechanisms in the energy substrate switch, in the hypertrophied failing heart, involves deactivation of the PGC-1α/PPARα complex at both transcriptional and posttranscriptional levels [36]. Changes in gene expression in the failing hearts include down-regulation of mitochondrial fatty acid oxidation and glucose metabolism enzymes and together this is consistent with observed metabolic alterations [37], as also detected in Study II and IV.

1.1.5.2 Structure/contractile proteins

In HF several contractile proteins (e.g. Myosin, Actin, Tropomyosin, Troponins and Titin) are impacted by transcriptional changes, and this causes a change in the composition of the myosin subunit. It is known that in hemodynamic overload in rodents, there is a shift from the α-myosin heavy chain (α-MHC) towards β-MHC. This switch leads to altered cross-bridge cycling kinetics, resulting in an increase in the economy of muscle contraction [38]. The importance of accurate expression of contractile proteins, and their exact alignment in sarcomeres, is underlined by the fact that mutations in sarcomeric proteins and e.g. Titin can cause cardiomyopathy [39-41]. It is also well known that the cytoskeleton is important in cardiac hypertrophy and HF. It is believed that accumulation of Tubulin, Desmin and several other membrane-associated proteins are compensatory mechanisms typical of HF, independent of the underlying cardiac disease [42]. Such increase in cytoskeletal proteins accompanied by a loss of contractile filaments and sarcomeric skeleton components may be regarded as the morphological basis of contractile and diastolic dysfunction in the failing heart.

In reaction to myocardial hypertrophy, an intricate series of changes in cellular and extracellular components are altered by changes in the extracellular matrix (ECM). The cardiac ECM is composed of 1) structural proteins, such as Collagen and Elastin; 2) adhesive proteins such as Laminin and Fibronectin; 3) anti-adhesive proteins such as Tenascin, Thrombospondin and Osteopontin, and 4) Proteoglycans [43]. Collagen and adhesive proteins bind to the cellular membrane through transmembrane receptors, such as the integrins. The interaction between adhesion proteins and cell membrane receptors ensure communication between the extracellular and intracellular environments [44]. Proteoglycans contribute to the architecture of the ECM, bind growth factors that participate in the paracrine cell to cell cross-talk, and promote tissue remodeling and cell migration [45]. Normally, ECM synthesis and degradation are tightly regulated, but during myocardial remodeling, ECM synthesis increases and/or degradation decreases to yield an increase in ECM, leading to fibrosis.

Matrix metalloproteinases (MMPs) are Ca^{2+}- dependent endopeptidases that maintain homeostasis of cardiac structure by digesting the ECM. The MMP family consists of more than 20 proteins and they have different substrates, which include collagenases (such as MMP-1 and MMP-13), gelatinases (MMP-2 and MMP-9), Stromelysin (MMP-3) and membranous type MMP (such as MT1-MMP). Most MMPs are inactive, secreted enzymes that act extracellularly after activation. However, the MMPS are regulated by a class of proteins called tissue inhibitors of metalloproteinases (TIMPs) and dysregulation of MMPs and TIMPs is

associated with various cardiovascular diseases and has been shown to be involved in hypertension and HF [46].

Increasing evidence suggests that binding of growth factors to the ECM is a major mechanism regulating growth factor activity. The ECM provides the architecture for multicellularity, whereas growth factors link ECM structures and molecules to the regulation of cell proliferation and differentiation. Growth factor signaling is not only genetically regulated inside the cell, but can also be modulated outside of cells by ECM proteins and enzymes.

1.1.5.3 Cell growth and maintenance

At the cellular level, pathological hypertrophy is accompanied by an increase in cardiomyocyte size, enhanced protein synthesis, reduced organization of sarcomeres, and re-induction of a fetal cardiac gene program that ultimately weakens cardiac performance. Numerous extracellular agonists and, in particular, those that act through G-protein-coupled receptors, such as α- and β-adrenergic agonists, endothelin, angiotensin, and 5-hydroxytryptamine, promote cardiac hypertrophy [47]. In addition, the complex molecular processes that lead to cardiomyocyte growth involve membrane receptors, second messengers, and transcription factors. The common final pathway of all these intracellular substances is gene expression, whose variations are now being revealed in increasing detail.

Currently, growth promoting factors such as Angiotensin (Ang-II), Endothelin (ET-1), members of the Interleukin-6 (IL-6) family of proteins, Insulin-like growth factor-1 (IGF-1), Nitric oxide (NO) and others have been identified as direct triggers of a hypertrophic response at the level of the cardiomyocyte [47]. Atrial natriuretic peptide (ANP) and Brain natriuretic peptide (BNP) oppose the hemodynamic actions of the renin-angiotensin-aldosterone system (RAAS) by enhancing renal electrolyte and water excretion. Normally expression of these peptides is up-regulated in cardiac ventricles in response to pathological hypertrophy. Circulating levels of both ANP and BNP positively correlate with ventricular dysfunction, with plasma levels of BNP better reflecting the severity of heart failure [48]. Both ANP and BNP also oppose the hypertrophic effect of Ang II and Aldosterone on cardiomyocytes via the Guanylyl cyclase-A (GC-A) receptor and Cyclic guanosine monophosphate (cGMP) generation [49].

The cardiac interstitium constitutes a reservoir of growth factors, locally synthesized and released by different cell types in the myocardium, under the effect of mechanical, hormonal and electrical stimulation. Growth hormone (GH) and growth factors like IGF-1 also play a

role in the development, growth and function of the cardiovascular system. It is believed that GH and IGF-1 activate several mechanisms that protect against the development of heart failure in the short term. On the other hand, a condition of GH excess can cause cardiac dysfunction, but these functions are not yet completely understood [50]. In addition, it is known that among growth factors, the Fibroblast growth factor (FGF) family, including FGF-1 and FGF-2, the Epidermal growth factor (EGF), the Vascular endothelial growth factor (VEGF), and IGF-1 are involved in hypertrophic effects of cardiac myocytes via autocrine and paracrine mechanisms. It is also known that other growth factors, such as Transforming growth factor β (TGF-β) and Platelet-derived growth factor (PDGF), modulate cardiac myocyte hypertrophy [51].

1.1.5.4 Cell signaling/communication

Recent evidence suggests that normal (and exercise-induced) cardiac growth is regulated in large part by the growth hormone/IGF axis via signaling through the Phosphoinositide 3-kinases (PI3K/Akt) pathway. In contrast, pathological or reactive cardiac growth is triggered by autocrine and paracrine neurohormonal factors released during biomechanical stress that signals through the Gq/phospholipase C pathway, leading to an increase in cytosolic calcium, activation of Protein kinase C (PKC), induction of immediate-early genes, re-expression of embryonic genes, and increased synthesis of contractile proteins [52]. In addition, a complex web of signaling pathways has been implicated in the transmission of stress signals leading to cardiac hypertrophy. Our Study II indicates differences in relation to these pathways in CAD and DCM.

1.1.5.4.1 Kinase signaling

Cardiac eutrophy and physiological hypertrophy are largely mediated by signaling through the peptide growth factors IGF-1 and GH. GH acts predominantly via increased production of IGF-1 [53]. When IGF-1, insulin, and other growth factors bind to their membrane tyrosine kinase receptors, the PI3K subgroup Iα is activated and phosphorylates membrane phospholipid phosphatidylinositol 4,5 bisphosphate [54]. This leads to recruitment of the protein kinase Akt (also known as protein kinase b) and its activator, 3-phosphoinositide-dependent protein kinase-1 (PDK-1), to the cell membrane via interactions between kinase pleckstrin homology domains and the 3′-phosphorylated lipid [55]. Accumulated data suggest that PI3K/Akt signaling transduces adaptive cardiac hypertrophy; e.g., a central role of the

p110α pathway in IGF-1 induced growth and normal and exercise-induced hypertrophy was demonstrated utilizing mice expressing constitutively active or dominant-negative mutants of PI3K specifically in the heart [56]. Strikingly, the adaptive hypertrophy seen with constitutive activation of cardiomyocyte PI3K does not progress into a maladaptive hypertrophy. Further, supporting a critical role for the PI3K/PDK1/Akt pathway in regulating normal heart growth is the finding that cardiac-specific inactivation of PDK1 leads to reduced cardiac growth and a cardiomyopathic outcome [57]. Finally, cardiac-specific inactivation of Phosphatase and tensin homolog on chromosome 10 (PTEN), a tumor-suppressor phosphatase that negatively regulates the PI3K/Akt pathway by dephosphorylating 3′-phosphorylated phosphoinositides, resulted in cardiac hypertrophy [58,59].

As noted above, a major kinase effector of PI3K signal is Akt, which is at a signaling cascade branch point. While its effects on cell death/survival are directly mediated via phosphorylation of the Forkhead box, sub-group O (FOXO) family of transcription factors and other regulators of apoptosis [55], it is the two signaling branches downstream of Akt, not Akt itself, that largely determine the nature of a given hypertrophic response. One branch leads to mammalian target of Rapamycin (mTOR) and the protein synthetic machinery, which is essential for all forms of hypertrophy [60]. The other branch leads to Glycogen synthase kinase-3 (GSK-3), which also regulates the general protein translational machinery as well as specific transcription factor targets implicated in both normal and pathologic cardiac growth [61]. In addition, activity of both of these branches can also be regulated by stress activated, Gq-dependent mechanism that are independent of Akt.

The heterotrimeric G-proteins Gq and G11 are functionally redundant transducers of phospholipase C signaling from prohypertrophic heptahelical receptors for angiotensin, endothelin, norepinephrine, and other neurohormones [62]. PKC- and inositol 1,4,5-triphosphate (IP3) mediated calcium release are considered to be the major effectors of Gq signaling. However, PI3K-dependent signaling is also activated by this pathway but differs from physiological PI3K signaling in that the activated PI3K isoform (γ) is distinct from that activated by IGF-1. The mechanisms of its activation also differ, whereas p110α is activated via tyrosine phosphorylation by ligand-occupied growth factor receptors, p110γ is activated by recruitment to the sarcolemma by βγ subunits of activated Gq/G11, providing access to membrane phosphoinositides [54,62]. Strikingly, while p110α is required for normal or exercise-induced growth, but not pathologic stress-induced growth [63], p110γ is required for stress-induced hypertrophy, but not for normal growth [58,64]. Thus, PI3K signaling,

including that of Akt and both arms of its downstream signaling pathways (mTOR and GSK-3), is activated in response to both physiological and pathologic stimuli, and either branch downstream of Akt can regulate adaptive and maladaptive growth.

1.1.5.4.2 Calcium cycling

It is well known that HF is characterized by a down-regulation in gene expression and activity of the Sarcoplasmic reticulum calcium ATPase (SERCA) [65]. Mutations in Phospholamban (PLN), an inhibitor of SERCA, can cause dilated cardiomyopathy in humans, by preventing Phospolamban phosphorylation, leading to constitutive SERCA2a inhibition [66]. In addition, alterations in the phosphorylation status of Sarcoplasmic reticulum (SR) calcium release channel (Ryanodine receptor (RyR)) are observed in failing hearts [67]. Together, these findings support the hypothesis that abnormalities in calcium handling play an important role in development of HF. The main events of calcium cycling in myocytes are illustrated in Figure 1.

The sodium-calcium exchanger (NCX), which extrudes calcium from the cytosol in diastole, is up-regulated in HF, and is thought to be a counter regulatory process to reduction in SERCA [68]. In addition, the Plasma membrane calcium ATPase (PMCA), which transports calcium out of the cell, has been related to hypertrophy response. It is believed that in humans, SERCA accounts for approximately 70% of Ca^{2+} removal from the cytosol, NCX is responsible for 28% and PCMA and mitochondria remove just 1-2% of cytosolic Ca^{2+}. Its major functions are in the regulation of nitric oxide (NO) production in the myocardium and in leading signal transduction through the caveolae (structures in the cell membrane that carry a variety of receptors) [69].

The result of all these changes in Ca^{2+} related proteins is a reduction in peak systolic calcium, and an elevation and prolongation in diastolic calcium, resulting in reduced systolic contraction and a delay in diastolic relaxation, as well as impaired coupling of the calcium release.

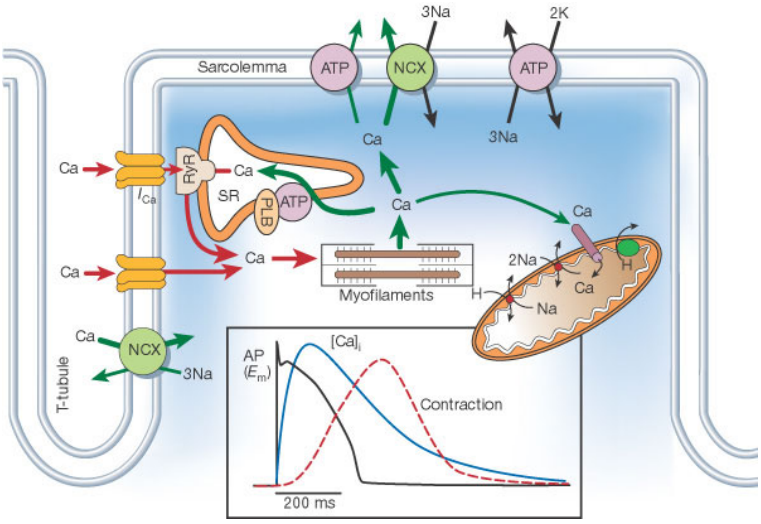

Fig. 1. Role of Ca^{2+} ions in regulation of cardiac excitation-contraction.
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1.1.5.4.3 Ion transporters

In addition to changes in Ca^{2+} handling mechanisms, electrophysiological remodeling in HF is characterized by major changes in ion channel function and expression of several ion channels, pumps and exchanger proteins, which alter the electrical phenotype and predispose to the development of lethal HF. For example, it is well-known that cardiac Na^+/H^+-exchanger (NHE) activity is up-regulated in several in vivo and in vitro models of cardiac pathological hypertrophy [71,72]. Elevated NHE activity depletes the transmembrane Na^+ gradient, which leads to increased intracellular Ca^{2+} mediated by the NXC (reviewed by Cingolani et al. [73]) and consequent activation of several signaling cascades (reviewed in Frey et al. [47]). Accordingly, inhibition of NHE by its specific inhibitor cariporide has been demonstrated in several studies [72,74-76] to "rescue" several models of cardiac hypertrophy in vivo. Because NHE inhibition does not appear to be associated with adverse hemodynamic consequences, this approach is a potentially interesting anti-hypertrophic treatment option. Several other ion transporting mechanism have also been suggested to be potentially novel targets in e.g. anti-arrhythmic therapy [77].

1.1.6 pH regulation in cardiomyocytes

It has been known for long that intracellular pH affects physiological processes profoundly (e.g. the contractile function), and a close control of hydrogen transport is therefore important. In cardiac myocytes several mechanisms maintain intracellular pH within a narrow range and

intracellular pH in myocardial cells is governed by the balance among four main sarcolemmal acid-equivalent ion transporters. The Na^+/H^+ exchanger (NHE) and the Na^+/HCO_3^- (NBC) co-transporter act to increase intracellular pH in acidosis [78-80] and the Na^+-independent Cl^-/HCO_3^- exchanger (anion-exchanger, AE) and Cl^--OH^- exchange (CHE) reduce pH in alkalosis [81]. The NBC cotransporter seems to play the main role in regulation of intracellular pH close to normal range (7.05-7.24), whereas at more acidic conditions, the NHE is the main proton extruding mechanism. All these pH regulating mechanisms are known to be under hormonal regulation [82] and it is important to note that most neurohormones that induce a positive inotropic effect in the heart, in some way also affect the pH regulation system. In addition to the four main transporters, a third proton-extruding mechanism has been identified in rabbit cardiomyocytes, as a vacuolar proton ATPase (VPATPase), which is activated during acidosis [83]. The VPATPase probably acts to attenuate the reduction in intracellular pH in cardiomyocytes exposed to metabolic inhibition. It is also believed that other mechanisms are able to compensate for increased H^+ load [83]. For example, lactate traverses the myocyte plasma membranes via a facilitated monocarboxylate transporter (MTC) system that functions as a proton symport [84] and Johannson et al. [85] showed that monocarboxylate transporter 1 (MCT1) protein level was up-regulated in cardiomyocytes from chronic heart failure rats, which indicates that this system may help protecting the myocytes from acidosis. In Study I, we showed that H^+/K^+-adenosine triphosphatase (H^+/K^+-ATPase or proton pump) was expressed and regulated in rat cardiac myocytes, both at the transcript and protein level. Functional in vitro studies indicated that the H^+/K^+-ATPase may account for up to 25% of the K^+-uptake in the ventricular cardiomyocytes. These findings indicate that the H^+/K^+-ATPase may share a pH_i regulating role with the NHE1. In contrast, a recent publication by Kemi et al. [86] concludes that H^+/K^+-ATPase does not contribute significantly to pHi maintenance. However, the complete role of the H^+/K^+-ATPase in pH-regulation and/or cell volume regulation has not yet been determined.

1.2 Functional genomics

Functional genomics is a field of molecular biology that attempts to make use of the wealth of data produced by genomic projects (such as genome sequencing projects) to describe gene (and protein) functions and interactions. Unlike genomics and proteomics, functional genomics focuses on the dynamic aspects such as gene transcription, translation, and protein-protein interactions, as opposed to the static aspects of the genomic information such as DNA sequence or structure. Functional genomics uses high-throughput techniques mostly to characterize the abundance of gene products such as messenger ribonucleic acid (mRNA). Because of the large quantity of data produced by these techniques and the need to identify biologically meaningful patterns, bioinformatics is crucial to this type of analysis.

1.2.1 From a single gene approach to genome wide gene expression analysis

The sequencing of the entire human genome [87,88] has opened a new era in biomedical research in which gene identification and cloning is not pursued in the same scale as previously. The challenge is now to identify the function of the products of the genes in vivo, the diseases in which each gene is involved, and the therapeutic benefits to be gained from this information. Already, the use of gene expression analyses and gene array technology, together with advancements in proteomics, are beginning to be used to analyze e.g. genetically modified mice to determine “upstream” and “downstream” factors involved in the function and mechanism of action of a particular gene product. This is an important step towards the discovery of new diagnostic tools or novel drugs for the treatment of disease [89].

Microarray technologies have developed rapidly during the last decade and have changed the face of science. Today, microarray technology can be used for screening thousands of transcripts or single nucleotide polymorphisms (SNPs) or copy number variation or proteins or other biological components [90] in one single experiment. By conventional methods, this type of analysis would have taken several years.

1.2.2 Microarray technology

The history of microarrays begins more than 25 years ago with the Southern blot, which introduced the basic technique of anchoring nucleic acids to a solid support for analysis by hybridization [91]. Modern microarray analysis was for the first time introduced in 1995 by a Stanford research team led by Pat Brown and Ron Davis [92]. The authors described the use of a robotic system to spot deoxyribonucleic acid (DNA) oligonuclotides onto a glass slide in

ordered arrays, generating microarray slides. Only 45 oligonucleotide sequences were spotted on this first microarray, but the work initiated many new experiments and soon whole genomes of species such as yeast, bacteria, mice and humans were being spotted onto glass slides. In the same period of time when Brown et al. made the first microarrays at Stanford, Fodor et al. [93,94] invented a method for manufacturing microarrays by using photolithography for in situ synthesis of DNA probes on a silica wafer (commercialized by Affymetix Inc. (Santa Clara, CA)). This method was based on the principle that a set of oligonucleotide DNA probes (each approximately 25 nucleotides in length) is defined, based on its ability to hybridize to complementary sequences in target genomic loci or genes of interest.

1.2.2.1 DNA microarrays

A DNA microarray is a glass slide with attached DNA probes representing many genes arranged in a regular pattern. There are two major forms of DNA microarray technology:

I. Complementary DNA (cDNA) arrays, where the probes are PCR products (200 ~ 2 000 base pair long) obtained from cloned cDNA libraries, printed by a robot, and immobilized on e.g. an aminosilane coated slide. Microarrays made from PCR-amplified cDNA clones are highly specific and produce strong signals because of the extended length of the cDNA. However, where sequence information is available, as in now often the case for most organisms, long oligonucleotides offer similarly strong signals and good specificity. Oligonucleotide arrays are now therefore replacing cDNA arrays for RNA based expression analysis. Each cDNA array is usually hybridized with two samples (two-color system), including an experimental and a control/reference sample.

II. Oligonucleotide arrays, consists of oligonucleotide (20 ~ 80 mer oligos) probes that are synthesized either in situ (on chip) or by conventional synthesis, followed by immobilization on the array surface. This method, “historically” called DNA chip, was developed by Affymetrix, Inc. [93,94], but today there is a large variety of different oligonuclotide array formats. Each oligonucleotide array can be used with either two samples (two-color system) or only one sample (one-color system) (Figure 2.).

The original DNA microarrays were used exclusively for expression analysis, but today oligonucleotide arrays are also used to identify sequence variations like SNPs, for genotyping and to resequence gene products [95].

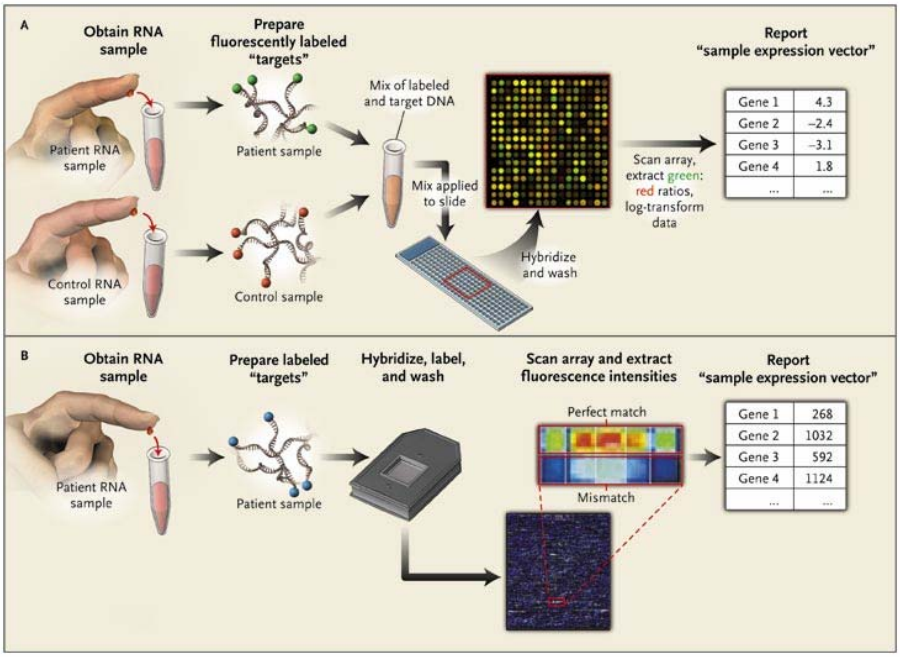

Figure 2. Overview of DNA Microarray Analysis.

Panel A: In a two-color analysis, RNA samples from e.g. patients and control subjects are individually labeled with distinguishable fluorescent dyes and hybridized to a single DNA microarray consisting of individual gene specific probes. Relative levels of gene expression in the two samples are estimated by measuring the fluorescence intensity for each probe, a sample expression vector summarizes the level of expression of each gene in the sample obtained from a patient. Panel B: A single color analysis, performed with the use of Affymetrix GeneChip. Labeled RNA from each biological sample is hybridized to a single array in which a series of gene-specific probes are arrayed. Gene-expression levels are estimated by measuring the hybridization intensity for a series of "perfect match" probes, and the background is measured with the use of a corresponding set of "mismatch" probes. Gene-expression levels are reported for each sample as a sample expression vector that summarizes the difference between the signal and background for each gene.

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1.2.2.2 Experimental design for DNA microarray experiments

Good experimental design in a microarray project requires the same principles and practices that are part of any scientific investigation. Appropriate controls are the foundation to any experiment. Forethought and consultation on the correct statistical practices and procedures for the design are always advantageous.

Optimizing design based on the experimental goal is an important part of a successful microarray experiment. One question that may be asked before designing an experiment is how much statistical power you wish to have to detect differentially expressed genes. This will determine the number of replicates needed [97]. Another question is what are the most

important samples, or comparisons you want to make, and how many experimental factors will be involved. For single-channel array experiments, it is obvious that more replicates should be done for samples of greater importance. For two-color array experiments, the many possible choices for designs pose a more complex problem which will be discussed in the following.

1.2.2.2.1 Replication

Replication is necessary in order to apply a statistical test and reduce variability inherent in microarray experiments. Replication falls into two categories; biological replicates and technical replicates [98]. In order to achieve results with any statistical confidence it is suggested that at least 3 biological replicates are used. Depending on the degree of intrinsic biological variation in the system, this may or may not be sufficient [99,100]. Technical replicates can be performed by using multiple arrays per sample. A second type of technical replication is dye-flip hybridizations (Figure 3.). A third type of technical replication is spot duplication on the slides where the conformity between the duplicate spot intensities can be used as a good indicator of the quality of the slides and the hybridization. However, biological replicates are more important than technical replicates [101]. So typically, a researcher should use biological replicates to validate generalization of conclusions and technical replicates to reduce the variability of these conclusions.

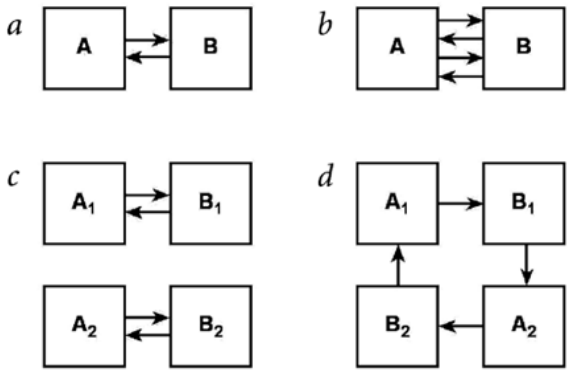
1.2.2.2.2 Design alternatives

The ability to make direct comparisons between two samples on the same microarray slide is a unique and powerful feature of the two-color microarray system. However, it is often impractical to make all possible pair-wise comparisons among the samples, because of cost or limitations in the amount of sample. Thus, an important step in designing an experiment is to decide how many technical replicates will be measured and how these will be paired together on arrays. The efficiency of comparisons between two samples is determined by the length and the number of paths connecting them [98,102]. It is most efficient to make the comparisons of greatest interest directly on the same array. Contrasts between samples that are never directly compared in an experiment are possible, provided that there is a path of comparisons linking them.

A commonly used means of indirect comparison for microarray experiments is a reference design (Figure 3.). This design uses a common reference RNA. The intensity of hybridization

of a test RNA sample to a given spot is compared to the intensity of hybridization of the reference RNA to the same spot. An advantage of this method is that as long as the amount of reference sample is not limiting, the design can be extended to handle large numbers of samples, and in class discovery experiments samples from a new class can be added and analyzed at a later stage [103].

I



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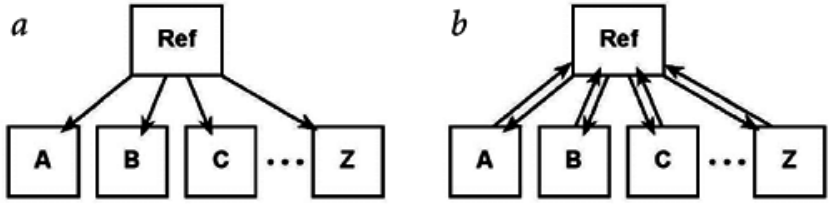

Figure 3. Experimental design.

Panel I. Experimental designs for the direct comparison of two samples. Boxes represent RNA samples that are labeled as varieties A or B. Subscripts indicate the number of independent biological replicates of the same treatment. Arrows represent hybridizations between the RNA samples and the microarray. The sample at the tail of the arrow is labeled with red (Cy5) dye, and the sample at the head of the arrow is labeled with green (Cy3) dye. The figures show a dye swap (*a*), a repeated dye swap (*b*), a replicated dye swap (*c*) and a simple loop design (*d*).

Panel II. Experimental designs using a reference RNA sample. Boxes represent RNA samples, and arrows represent microarrays, as in panel I. Panel *a*: the standard reference design uses a single array to compare each test sample (A, B, C, and so on) to the reference RNA. Panel *b*: a variation, with a dye swap for each comparison.

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Other design alternatives may be loop designs (Figure 3.) or factorial designs. The simple loop design can be an efficient alternative to the reference design [98,104]. However, the estimation efficiency of a simple loop is greatly reduced by loss of just a single array [105]. The previous types of designs have been single factor experiments. Experiments investigating two or more factors require a more complex design [106]. A key premise is that it is possible to define an *a priori* number of contrasts that are of specific interest. The approach is then to design experiments that provide maximal information for these contrasts.

1.2.2.3 RNA preparation, labeling and hybridization

1.2.2.3.1 RNA quality

The first and most critical step in sample preparation for DNA expression array analysis is isolation of total or mRNA from the experimental samples. The purified RNA should always be visualized by denaturating gel electrophoresis to verify the integrity of the ribosomal bands. This can be done by conventional gel electrophoresis [107] or by capillary electrophoresis e.g. with the Bioanalyzer (Agilent Inc. Palo Alto, CA) [108]. It is believed that the 18S to 28S ratio should be at least 1.8 and the RNA Integrity Number (RIN) [109] (obtained from the Bioanalyzer software) value above 7 to ensure good RNA quality [109]. In addition to gel-electrophoresis it may be useful to measure the RNA integrity, quality and quantity by a spectrophotometer. The ratio absorbance at 260 and 280 nm is used to assess the purity of RNA. A ratio of ~2 is generally accepted as "pure" for RNA. The 260/230 ratio should commonly range from 1.8-2.2, and if the ratio is appreciably lower, this may indicate the presence of co-purified contaminants. If the RNA is degraded or contaminated, it will not be usable for labeling. For such analyses the NanoDrop (NanoDrop® Technologies Inc. Wilmington, De), a cuvette free spectrophotometer is useful.

1.2.2.3.2 Labeling

RNA extracted from biological samples is typically labeled with fluorescent dyes. The commercial cyanine dyes Cy3 and Cy5 are the most commonly used dyes in labeling reactions [110]. Fluorescence labeled samples can be prepared by several different methods including direct or indirect cDNA labeling [111-113] (Figure 4.).

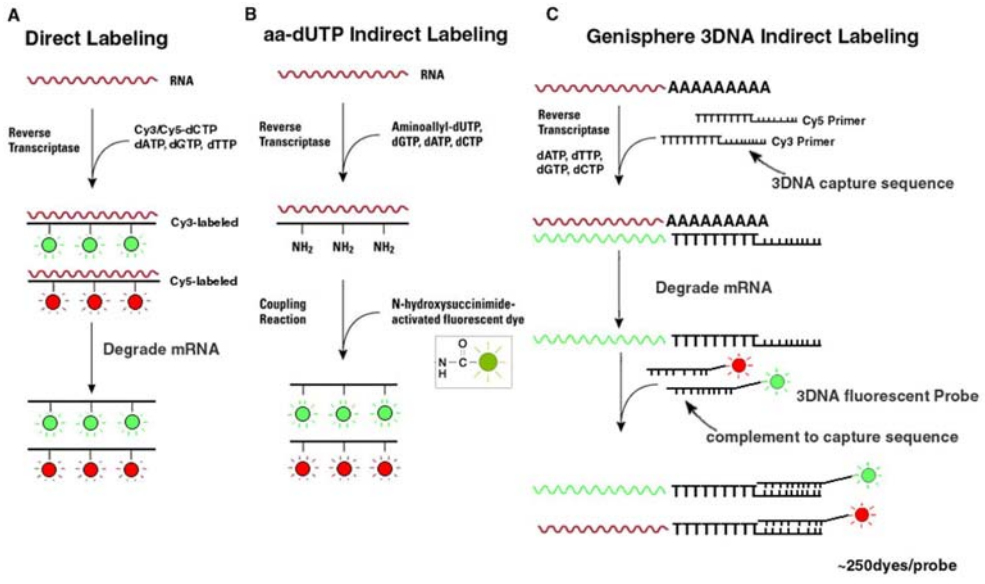

Figure 4. Microarray target labeling procedures.

The figures show a schematic representation of different experimental labeling procedures. Panel A: Direct labeling. Panel B: Aminoallyl indirect labeling. Panel C: Genisphere 3DNA labeling.

Adapted and reprinted by permission from Molecular Vision: Yu J, Othman MI, Farjo R, Zareparsi S, MacNee SP, Yoshida S *et al*.: Evaluation and optimization of procedures for target labeling and hybridization of cDNA microarrays. *Mol Vis* 2002, 8:130-7.: 130-137. [111], copyright 2002.

In the direct cDNA labeling method [114], fluorescence modified deoxynucleotides are incorporated during the first strand cDNA synthesis from an RNA template using reverse transcriptase. Although this method is relatively easy to perform, fluorescence modified nucleotides are bulky and Cy5- and Cy3-modified nucleotides may incorporate with different efficiency. In the indirect cDNA labeling method, e.g. aminoallyl-modified nucleotides are incorporated during reverse transcription reaction, and fluorescent dyes are subsequently coupled to the reactive amino groups in the cDNA. A different indirect method (3DNA by Genisphere Inc. Hatfield, PA) uses fluorescent dendrimer complexes to label cDNA [115]. After cDNA synthesis, a fluorescent dendrimer with hundreds of dye molecules per complex is hybridized to the cDNA. Affymetrix uses a different detection scheme than previously described, where mRNA is reverse transcribed into cDNA and then copied into biotinylated complementary RNA (cRNA) [116]. The biotin-streptavidin complex is then used to add the fluorescent tag.

1.2.2.3.3 Hybridization

In a two color experiment, two labeled samples (unusually a “control” or a ”reference” sample and the sample of interest) prepared from two RNA sources are co-hybridized to the same DNA microarray (for some of the oligonucleotide array formats e.g. Affymetrix, only one sample is hybridized to each array). The conditions during this step must be optimized to promote specific binding of labeled samples to its target probes and to reduce background. Important parameters include hybridization temperature, length of hybridization, concentrations of salts, pH of the hybridization solution, and the presence or not of denaturants such as formaldehyde in the hybridization buffers [117]. During hybridization the arrays must be stored in a humidified, temperature controlled, dark environment. Small, affordable chambers that house one array work well, and can simply be placed in a standard incubator or water bath during the hybridization. However, today most users use automatic hybridization stations, where several arrays can be hybridized simultaneously and e.g. agitation can be applied to the samples during hybridization. Automatic hybridization stations have increased the quality of hybridization and post-hybridization wash and usually the specificity of the hybridization signal is increased and the background noise reduced.

1.2.2.3.4 Scanning and image analysis

After hybridization, arrays are typically scanned with an instrument that uses lasers as a source of excitation light and a photomultiplicator as detectors. This detection method allows determination of fluorescence from each of the labeled samples [118]. After scanning, image analysis must be performed to acquire target signal intensities [119]. Typically a program like GenePix (Axon Inc., Sunnyvale, CA) is used for image analysis of microarray data. Many image processing approaches have been developed [120-124], among which the main differences relate to procedures for spot segmentation (how to distinguish foreground from background intensities) [125,126]. Further analysis includes procedures like filtering, normalization and statistical analysis for finding differentially expressed genes or methods for clustering the genes or samples with similar expression patterns/profiles.

1.2.2.4 Reporting microarray results

Microarray studies generate large amounts of data which can not be published in journals. However, these data may be valuable to other researchers, so microarray data should be made publicly available. There are two main public repositories for microarray data: ArrayExpress

[127] at European Bioinformatics Institute (EBI) and Gene Expression Omnibus (GEO) [128] at National Center for Biotechnology Information (NCBI). These two and the proprietary repository Center for Information Biology gene Expression (CIBEX) [129] are recommended by the Microarray Gene Expression Data (MGED) society [130].

To compare data across experiments performed at different times and in different laboratories, all information related to the microarray experiments must be reported in a common way, using a widely accepted form. Such a form is called Minimum Information About Microarray Experiments (MIAME). The MIAME standard outlines the minimum information that should be reported about microarray experiment to enable its unambiguous interpretation and reproduction [131]. The MIAME includes a detailed description of the following six sections: experimental design, array design, samples, hybridization, measurements (raw expression data), controls [131]. Today most journals require microarray data to be MIAME compliant and that the raw data are submitted to a public repository [132,133].

1.2.3 Data collection and data analysis

1.2.3.1 Data pre-processing and normalization

Before it is possible to extract knowledge from microarray data, the raw data must be pre-processed and normalized. The data extracted by image analysis must be pre-processed to exclude poor-quality spots and normalized to remove systematic errors before downstream analysis.

Some commonly used methods for calculating normalization factor include: global normalization that uses all genes on the array, non-linear normalization method (lowess)[134] and internal controls normalization that uses known amounts of exogenous control genes added during hybridization [135-138]. In most cases the non-linear normalization method which corrects for dye basis of gene intensity and spatial information, is believed to be superior to the other methods. In addition, if there is a significant difference in the distribution of log-ratios among the print-tips, suggesting a possible spatial effect, print-tip group lowess normalization should be considered [137]. Apart from within a single array, the distribution of gene expression ratios from replicate experiments might have different distribution of log ratios. Therefore scaling adjustment may be necessary to standardize the distribution of log-ratios across replicate experiments to prevent any particular experiment from becoming dominant and affecting downstream statistical analysis [135].

For global and lowess normalization a comparable global gene expression is expected between the two samples. If there is a global shift between the samples these normalization methods will bias the results. In such cases normalization by internal controls (spikes) is a good solution [139-141]. External spike mRNAs may be added to the samples in predefined ratios and the normalization can be done by adjusting the observed ratios compared to the theoretical ratios [142-144].

1.2.3.2 Finding differentially expressed genes

Fold change (FC) was the first method used to evaluate whether genes are differentially expressed, and is a reasonable measure of effect size. However, it is widely considered to be an inadequate test statistics because it does not incorporate variance and offers no associated level of confidence [145]. Another method of identifying differentially expressed genes it the use of a two-sample *t*-test (or one of its nonparametric equivalents, e.g., Mann-Whitney U test) to calculate *p*-values [146]. Basically, this step involves statistical hypothesis testing where a null hypothesis (the nonexistence of differential expression) is contrasted to an alternative hypothesis (the existence of a differential expression). The calculated *p*-value represents a threshold for which the null hypothesis is rejected (the alternative hypothesis is accepted), and therefore observed differences in gene expression are statistically significant. In addition, identifying genes that are differentially expressed across more than two samples can be achieved in a way similar to what was described above, but using different tests, for example, ANOVA (analysis of variance) or equivalent nonparametric tests such as Kruskal-Wallis analysis of ranks [146]. ANOVA is common tool for studying data from experiments with multiple categorical factors [147]. The ANOVA model accounts for multiple sources of variation in microarray experiments. Significance analysis of microarrays (SAM) [148] is another approach that has been used in many studies. SAM identifies genes with statistically significant changes in expression by identifying a set of gene-specific statistics (similar to the t-test, thus taking into account both magnitude of change and variability of expression) and a corresponding false discovery rate (similar to a P value adjusted for multiple comparisons). Several other methods based on e.g. regression modeling [149], the empirical Bayes method [150], and the mixture model [151] have also been used for finding differentially expressed genes from microarray experiments.

1.2.4 Knowledge discovery

1.2.4.1 Learning

Soon after microarrays were introduced, many researchers realized that learning techniques could be used to discover new subclasses in disease states [152,153], to identify biomarkers associated with disease and even that the expression patterns of the genes could be used to distinguish subclasses of disease [154-156].

Many different learning algorithms are today extensively used in microarray research. The different algorithms are used either to discover new categories within a data set (class discovery; unsupervised learning) or assign cases to a given category (class prediction; supervised learning/classification) [157].

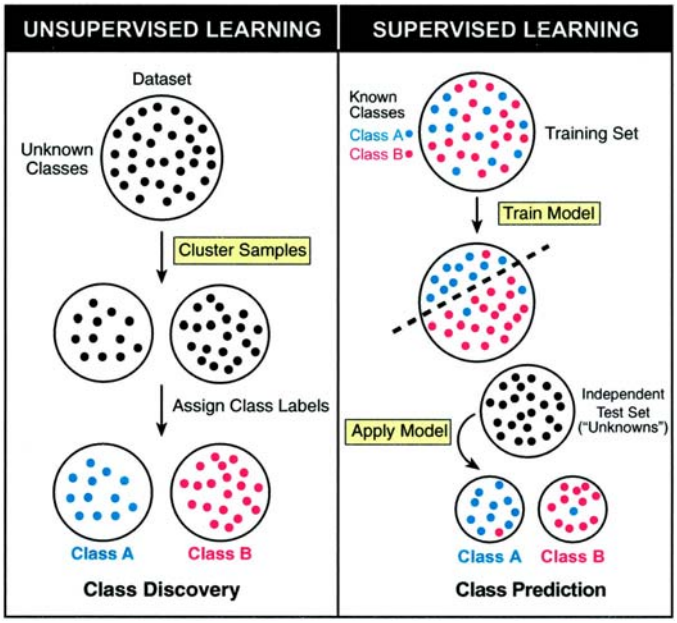

Figure 5: Unsupervised versus supervised learning. In unsupervised learning multiple samples are clustered into groups based on overall similarity of their gene expression profiles. This approach is useful for discovering previously unappreciated relationships. In supervised learning multiple samples from different known classes are used to train a model capable of classifying unknown samples. This model is then applied to a test set for class label assignment.
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1.2.4.1.1 Unsupervised learning

Algorithms for unsupervised learning or cluster analysis group objects on the basis of some sort of similarity metric that is computed for one or more "features" or variables. For example, genes can be grouped into classes on the basis of the similarity in their expression profiles across tissues, cases or conditions. Hierarchical cluster analysis graphically presents results in

a tree diagram (dendrogram), and is probably the most common unsupervised learning algorithm in microarray analysis [158]. Its popularity is understandable because no hypotheses and almost no data assumptions are required, but the researcher is quite certain to obtain a clustering of samples or genes, irrespective of sample size, data quality or experimental design or indeed any biological validity that is associated with the clustering.
Non-hierarchical clustering methods divide the cases (samples or genes) into a predetermined number of groups in a manner that maximizes a specific function (for example, the ratio of variability between and within clusters) [159].

1.2.4.1.2 Supervised learning

In supervised learning (often called "classification", "class assignment", "class prediction" or "class discrimination"), the aim is to obtain a function or rule that uses expression data to predict whether a case is of one type or another (for example, drug-resistant versus non-drug-resistant). For example, a rough set algorithm finds the rule that best classifies a set of available cases for which the correct type is known [160]. First the samples are divided into groups based on e.g. a clinically relevant parameter such as disease aetiology, prognosis, or response to therapy. Then a molecular signature is created by choosing genes whose expression is solidly associated with the parameter in question, by weighting genes based on their individual predictive strengths.
Algorithms are typically developed on a "training" data set and evaluated on an independent "test" data set. The requirement for both the "training" and "test" data set is that the categories to which objects belong are known. Many supervised classification algorithms are available. The great challenge is to determine the optimal degree of model complexity that a given data set can support. A common misconception is that the set of the most differentially expressed genes will necessarily give the best predictive accuracy. The gene list that is obtained from hypothesis testing does not necessarily give the best prediction. No one method for constructing prediction algorithms is widely accepted as superior or optimal. However, experience suggests that with the sample sizes that are typically available in microarray studies, simpler methods might out-perform more complex approaches.

1.2.4.2 Gene annotation

A "gene annotation" is a "comment" attached to a gene or gene product, which can be a protein. The comments can, for example, be a DNA or peptide sequence, describe the

biological functions of the protein, its interactions with other genes/proteins, and the metabolic pathways in which the protein is active. In microarray experiments, after the genes of interest have been identified using statistical tools, their annotations should be acquired, in order to make inferences about the validity and biological interpretations of the findings, and to generate new hypotheses.

1.2.4.2.1 Retrieving annotations from public databases

The meta-analysis of microarray data sets largely depends on rapid access to previously described annotations of the genes being studied. Today, diverse publicly available resources exist that catalog various attributes of genes, ranging from their mapped coordinates within the genome to the enzymatic function of the proteins they encode. These include GenBank [161], UniGene [162], Entrez (LocusLink) [163], SwissProt [164], Online Mendelian Inheritance in Man (OMIM) [165], PubMed [162], as well as many others. Although these resources are highly informative individually, the collection of available content is of higher value when provided in a unified and indexed in a robust manner. Several web-based database tools like, SOURCE [166], GeneCards [167], GeneCruicer [168], NetAffx [169] and *GeneTools* [170] (Study III) are now available. These tools are designed to bring together annotation information from a broad range of resources, and provide it in a manner particularly useful for genome-scale analyses, which means that lists of gene reporters can be submitted and the user receives annotation information for all these in one query.

1.2.4.2.2 Gene ontology

The Gene Ontology (GO) project was founded to advance the development and utilization of bio-ontologies and semantic standards for molecular biology [171]. The GO is divided into three sub-ontologies: molecular function (MF), biological process (BP), and cellular component (CC), to describe attributes of gene products or gene product groups. Briefly, MF describes what a gene product does at the biochemical level. BP describes a broad biological objective. CC describes the location of a gene product, within cellular structures and within macromolecular complexes [171].

The GO ontologies are structured vocabularies in the form of directed acyclic graphs (DAGs) that represent a network in which each term may be a “child” of one or more than one “parent”. Relationships of child to parent can be of the “is a” type or the “part of” type. The “is a” type refers to when a child is an instance of the parent. For example, nuclear

chromosome “is a” chromosome. “Part of” is slightly more complex. For example, A “part of” B means that whenever A is present, it is always a part of B, but A does not always have to be present. An example can be nucleus “part of” cell. Nuclei are always part of a cell, but not all cells have nuclei.

The annotations of gene products to the GO vocabularies are attributed to a source, which may be a literature reference, another database, or a computational analysis. The annotations include not only the source attribution, but also an indication of the evidence on which the annotation is based. A simple controlled vocabulary (evidence codes) is used to describe the evidence supporting the attribution, such as “traceable author statement” or “inferred from direct assay”. Referencing each annotation with both experimental method and citation is intended to help researchers evaluate the reliability of an annotation and is critically important to the future evaluation and use of these annotations [172]. The ontologies and annotations are provided publicly as part of the GO database resource (http://www.geneontology.org/).

1.2.4.3 Gene-class testing

Among the most widespread applications of GO data is the use of GO terms and gene product annotations to help interpret the results of microarray experiments. Correlation between the functional information captured by GO and the expression patterns of a set of genes (gene-class testing) can help to highlight underlying biological phenomena. Comparisons of gene lists are important in order to answer questions such as “are genes involved in process P overrepresented among the total of differentially expressed genes in an experiment” or “does treatment A induce more genes involved in process P than treatment B?”.

Several software tools have been developed to facilitate the analysis of gene expression data using GO (www.geneontology.org/GO.tools.microarray.shtml), and some papers reviewing the relative merits of a subset of these tools have recently been published [173-175]. These tools are suited for analysis of the GO hierarchy and for statistical evaluation of GO category representations between gene lists [176]. Several different statistical tests are offered through the different tools, and suit different situations/questions.

2. Objectives

The main purpose of this project was to investigate molecular mechanisms of myocardial hypertrophy and heart failure in experimental models and clinical samples. We aimed to establish and use microarray technology and bioinformatics tools to obtain these results.

The specific objectives of the studies were

Study I

To explore the presence and localization of H^+/K^+-ATPase gene and protein expression in the rat heart, and to investigate whether the enzyme could contribute to potassium transport across the sarcolemma.

Study II

To use gene expression profiling, gene-class testing methods built on functional annotations (GO) and supervised classification to identify aetiology-specific biological processes and potential molecular markers (classifiers) for different aetiologies of end-stage heart failure.

Study III

To develop a general gene annotation tool that could handle information from microarray studies. Important features should be user friendliness, recent updates of annotations, options to add user-defined annotations, and procedures for statistical hypothesis testing.

Study IV

To identify genes and gene-classes related to biological processes that are differentially regulated during hypertrophy development and genes whose expression differ between pathological and physiological hypertrophy.

3. Methodical considerations

3.1 Animal models

Due to limited availability of human cardiac tissue, we chose animal models for cellular and molecular analyses of heart failure and hypertrophy mechanisms in Studies I and IV.

3.1.1 Rat myocardial infarction and heart failure model

The rat was chosen because the species has been extensively studied in cardiovascular disease and many of the similarities and differences to human physiology and pathophysiology are known. Adult female Sprague-Dawley rats were used to minimize physiological growth throughout the study period. In Study I and IV, infarctions were induced by ligation of the left coronary artery. This method is well established in our laboratory [75,177,178].

To verify myocardial infarction and to assess the extent of heart failure, left ventricle (LV) pressure and infarct size were measured by echocardiography. The myocardial infarction model is an established heart failure (HF) model with high prevalence of severe HF dependent on infarct size [179-181]. It has many similarities to human post-infarction HF and the coronary artery ligation induces a transmural myocardial infarction in the free wall, with infarction size dependent on the ligature positioning. Within few days the infarct area is macroscopically demarcated to the non-infarcted tissue. LV remodeling occurs in the rat model similarly to the human, except that scar formation is complete earlier (3 weeks vs. 4-5 weeks). After large myocardial infarctions, LV remodeling and cardiomyocyte hypertrophy is progressive and contractile function and calcium handling is impaired [182,183]. Dissimilarities between experimentally induced pathology and disease are usually marked. HF due to one large, well defined transmural infarction after permanent coronary artery ligation may differ significantly from clinical ischemic HF. Patients usually have diffuse CAD with recurrent infarctions and modern interventions frequently result in reperfusion of the infarcted area.

In Study I, we used LV pressure measurements to detect differences in contractile functions between sham operated animals and infarcted rats. It has been established that the maximum rate of rise of left ventricular pressure, dP/dt_{max}, is a sensitive measurement of acute changes in LV contractile function. LV dP/dt_{max} also correlates with basal contractility in failing and non failing hearts but is affected by pre-load, heart rate and hypertrophy [184]. Interpretation of dP/dt_{max} must therefore be done with these limitations in mind.

3.1.2 Rat exercise training program

To maximize cardiovascular adaptation and induce physiological hypertrophy in Study IV, we chose to use a custom made inclined treadmill running model, which allows us to have close control of exercise intensities and training adaptation. This model has been extensively characterized and used in our laboratory and described in detail by Wisloff et al. [185]. In this running model, the exercise sessions were carried out for 1.5 h/day, 5 days/week, in intervals of 8 min at 85-90% of maximal oxygen uptake (VO_{2max}). To reduce the speed, uphill treadmill running was performed. This results in a full body exercise that taxes the cardiovascular system maximally, and mimics physiological adaptations in humans [186,187]. Several studies from our laboratory confirm that hypertrophy is induced after 4 weeks with this training program by increased LV and RV weights and myocyte length [185,187,188]. In contrast to pathologic hypertrophy, our model of exercise training-induced hypertrophy is associated with improved contractile function and calcium handling in the cardiac myocytes [187,188]. Due to these characteristics, this rat treadmill exercise program serves as a good model for physiological hypertrophy.

3.2 Human samples

In Study II, tissue samples from transplant and normal hearts were used. Compared to e.g. the rat myocardial infarction model, the human infarcted myocardium samples were much more inhomogeneous. Typically, more scar tissue was included in the samples. For the RNA isolation we tried to avoid these areas of the tissue samples as much as possible. Among other factors, differences in age and degree of failure introduce more variability in the data generated in this study than for the rat model.

A second difference compared to the rat model is that the non-failing human subjects were significantly younger than the patients with failing hearts and most of the non-failing patients were females whereas the failing patients were males. However, this did not seem to influence the results and we were not able to connect the pattern of differentially expressed genes to gender. Despite this biological variation in the samples we were able to find expression patterns that were significantly different between failing and non-failing hearts, and between different aetiologies, such as CAD and DCM.

3.3 Gene expression measurements

3.3.1 Competitive rt-PCR

For the competitive reverse transcriptase polymerase chain reaction (rt-PCR), we cloned two PCR products; one from the rt-PCR and one from a PCR of genomic H^+/K^+-ATPase (competitor). These clones were purified, quantified and used as a standard curve and a competitor for each sample that were tested. In the rt-PCR reactions, we always included the "genomic" competitor and after gel electrophoresis the expression bands were related to the standard curve and corrected for the competitor. In this way we were able to assess RNA expression in a semi quantitative manner. This method did not allow us to measure the accurate number of transcripts expressed, but it was good enough to distinguish between different groups of samples compared. Several other studies in our group and elsewhere have used this method with success [75,177].

A limitation of this method is that we were not able to correct for different amounts of input RNA, but this could have been tested by using a "house keeping" gene as control. To compensate for this, we used several biological and technical replicates that give statistical power. In addition, we established a second assay for Nppa (ANF) expression where we, for these samples, confirmed known expression patterns. A second limitation of this competitive rt-PCR method is that a genomic competitor was used. By using an RNA competitor we would have been able to control every step from the cDNA synthesis and we think this would have increased the possibility to quantify the exact amount of expressed transcripts. However, this is not usually the main focus for researchers, who often need to have a method that can distinguish gene expression between groups of samples.

3.3.2 Real time PCR

In Study II we used real time PCR (RT-PCR) for verification of microarray results. RT-PCR is a more sensitive and easier method to perform, than competitive rt-PCR.

Several algorithms have been developed to calculate absolute quantitative measurements, but how good these really are is still debatable. We chose to use TaqMan chemistry for the RT-PCR reactions and for relative quantification we used a modified ΔΔCT equation [189]. For these assays we chose to use Beta-actin to normalize the RT-PCR expression ratios for each individual sample. What genes that can act as "house keeping" genes in a heart is not well known. Several studies have found that most genes commonly known as "house keeping" genes may be differentially expressed in different cardiovascular settings. We tested Beta-

actin in heart failure samples vs. non-failing samples and in different aetiologies of heart failure, and did not find any significant differences in gene expression between the biological samples. Moreover, these analyses showed a close correlation with the microarray results for a sub-set of genes.

Today, most reviewers of microarray manuscripts require RT-PCR verification of the microarray results. Several studies have been done and most of them conclude that microarray results and real time data correlate very well. Since these two methods measure gene expression at the same biological level, it has been concluded [101] that in some situations this type of verification is unnecessary. Functional verifications with enzymatic assays or protein measurements may therefore be more relevant. However, the reason that many reviewers still require verification is that in the early days of microarry technology, the gene probe quality was poor and the user could not be sure that he was looking at the gene he though the clone represented. This problem will be discussed in the next chapter.

3.3.3 DNA microarray analysis

3.3.3.1 cDNA microarrays

Only cDNA microarrys were available when the studies of the present project were performed. We used glass microarrays with cDNA probes (200-2.000 nucleotides in length) printed by the Norwegian Microarray Consortium (NMC). The clones for the cDNA probes were purchased from the IMAGE consortium [190]. Errors in the IMAGE collections of clones have been reported, and additional errors may have occurred during production. As a response to this, NMC resequenced a large proportion of the printed PCR products from both the human and rat libraries. They found that ~70-80% of the human clones and ~60-70% of the rat clones could be verified. Among the remaining 20-40% of clones, not all are likely to be "wrong". Some could be classified as "not verified" as a result of e.g. lack of successful sequence reactions. Uncertainty of the exact nature of probes is a general concern with cDNA microarrays and a strong argument for verification of microarray data with other methods. In Study II, we resequenced the most significant genes and all were found to be correct. In addition, we focused less on single genes, but more on functional groups (e.g. GO-classes) of genes that were differentially expressed between the studied groups. These strategies are well suited to increase reliability of our interpretations.

A second concern when using cDNA microarrays is cross-hybridization of the labeled sample to non-target homologous probe sequences on the microarray [191,192]. This cross-

hybridization is mainly due to hybridization between the poly(A)-tail of the sample mRNA and the cDNA probes which also contain poly dA/dT sequences. Different blocking solutions are used in order to prevent this unspecific cross-hybridization. We used standard oligo dT and cot-1 blocking. A new alternative is the LNA dT blocker from Genisphere. The potential problem with the use of dT blockers is that the concentration of PCR products on the array and between batches of arrays may vary. Therefore, it is difficult to find the optimal concentration of blocking reagents to use. However, methods for solving this have been developed (Bruland et al. paper submitted).

At the time of our first microarray experiments, we did not have full knowledge of this potential problem and just standard amounts of blocking reagents were used. Moreover, the variable length of the PCR product printed on the microarrays makes it difficult to find an optimal hybridization and washing temperature. We think this is the main reason for the observation of compressed expression ratios, compared to e.g. RT-PCR experiments.

Important issues are still the choice of microarray platform, labeling method and quality control, but it is now believed that most platforms individually work well (in good hands and with standardized protocols) and produce similar results (at least according to the representation of the function to the genes found), so local experience with the technology is essential [193-195]. However, there are still many problems to solve. Shields [196] has discussed some of those in a recent paper and suggest that it is time to go beyond MIAME to reach the desired level of confidence. What is needed is a proper evaluation of microarrays (including sample extraction and work-up, data handling and analysis) and an understanding of what is important to achieve consistent, accurate and reproducible results across laboratories. However, recently both US and European projects have been established to work with such issues.

3.3.3.2 Labeling methods

In the present studies (Study II and IV) we used a microarray labeling and hybridization protocol that enables the use of small amounts (1-5 μg) of RNA without amplification [115]. The 3DNA labeling system (Gensiphere INC.) provides a more predictable and consistent signal than direct labeling and than several of the other indirect labeling methods. There are two main reasons for this: First, the fluorescent dyes are a part of a 3DNA dendrimer, and it does not have to be incorporated during the sample cDNA synthesis. This avoids ineffective cDNA synthesis and unequal incorporation of the two dyes (e.g. Cy5 and Cy3), which can be

a problem in direct labeling methods. Second, because each 3DNA molecule contains the same number of fluorophores (approximately 350 for the Genisphere 350 3DNA kit), the signal generated from each sample cDNA molecule will be largely independent of base composition or length of the transcript. In contrast, the signal from direct and most indirect labeling methods will vary depending on the base composition and length of the sample cDNA generated during labeling.

In our studies (Study II and IV) and in other early microarray studies using the 3DNA method, compressed ratios are observed. In addition to possible unspecific binding, this appears to be a result of low dynamic range of the fluorescence signals obtained. Studies comparing different labeling methods confirm this [112,197]. Despite these limitations, we and others [143,198,199] have shown that the 3DNA method can identify differentially expressed genes, since we have obtained good results using external spikes, and since the RT-PCR verification results fit well with the obtained microarray results. After our Study I was finished, the 3DNA protocols were improved and new equipment has been made available. Among the improvements of the protocols is the introduction of a "two-step" hybridization (used in the exercise part of Study IV), where the sample cDNA first is hybridized to the array and then the fluorescent dendrimers are hybridized to the target sequences in a second hybridization before wash and scanning. In addition, Gensisphere has introduced the LNA blocking solution (described in section 3.3.3.1) which works well on cDNA arrays. Today most laboratories have automatic hybridization stations that ensure good mixing of the cDNA, constant and precise temperature under the hybridization, and stringent and equal wash conditions. Together, these improvements result in better signal to noise ratios, and an increased dynamic range of the signals.

3.3.3.3 Quality control

There is a mounting call for standards and quality control (QC) of microarray experiments. In every step, from sample preparation to data analysis, there is QC that can be performed. However, still good systems are missing for assessment of technical quality of the microarray experiments. Two characteristics of performance are very important, accuracy and precision. The assessment of these factors can be either for the purpose of technology optimalization or for the evaluation of individual hybridizations. Whereas accuracy refers to how close a measurement is to the real value, precision indicates how often a measurement yields the same result. When microarrays are discussed, the focus is often on precision, which relates to

reproducibility rather than accuracy. This is due to the fact that reproducibility is relatively easy to assess by performing repeated measurements. However, as discussed below, several types of external controls can be used for accuracy assessments of microrray data.

A very useful tool is external spike controls. Spike controls consist of exogenous RNA transcripts, which are added to the RNA samples prior to reverse transcription and labeling. For Study IV we used a commercial SpotReport Array Validation System (Stratagene, La Jolla, CA) to measure hybridization quality. To use this system, the arrays were spotted with probes for the exogenous transcripts. Different amounts of each exogenous transcript were added to the test and control sample to generate pre-defined ratios (10 different ratios) between the fluorescence signals for the two samples compared on a slide. In this way we were able to mimic differentially expressed genes. This information can be used to evaluate the reverse transcription, labeling and hybridization procedures, as well for assessing the dynamic range of the signal intensities. An extended spike set up can increase the value of using spikes as a QC method. An ideal set up may include 20-30 different spikes which are printed in all sub arrays. A wide distribution in the signal intensities (with log2 ratio 0, normalization controls), that covers the entire range of mRNA levels, will allow normalization using the spikes. This may be very important in analysis of microarray data if a global shift in gene expression is observed [200]. In addition, it is advantageous to include spikes at ratio levels that are both higher and lower than the range that is strictly required. So, in addition to include non-differential (normalization) controls, spikes designed to measure both low (two fold) and high (ten fold) differentials in a single experiment are useful.

3.3.4 Learning

Right from the beginning of microarray history, clustering and classification have been popular analysis methods. However, it is believed that unsupervised learning (class discovery) is overused. First, little information is available about the absolute validity or relative merits of clustering procedures [201,202]. Second, the evidence indicates that the clusters that are produced with typical sample sizes (<50) are generally not reproducible [203]. Third, and most importantly, unsupervised learning rarely seems to address the questions that are asked by biologists, who usually are interested in identifying differential expression. However, it is important to note that there might be cases where clustering is warranted, e.g., if the goal is to simply obtain a general description of how genes co-vary with respect to their gene expression levels within a population.

In supervised learning (class prediction), the aim is to obtain a function or rule that uses expression data to predict whether a case is of one type or another (for example, coronary artery disease vs. dilated cardiomyopathy as described in Study II). For example, a rough set algorithm finds the rule that best classifies a set of available cases for which the correct type is known. Because of this attempted optimization, over-fitting might be a concern. To estimate how well the rule will perform on fresh data, one should cross validate it on test data that are completely independent of the data from which the classification rule was derived, and there are many approaches to this [204]. One key point is the need to avoid selection bias. This requires cross-validation procedures that separate the validation data from all aspects of the rule derivation process, including the selection of initial transcripts to include in the model [205-207]. Early microarray papers failed to account for selection bias and thereby radically overestimated prediction accuracy. Effective cross validation requires an adequate sample size, and methods for estimating sample sizes for supervised learning studies have been developed [208-210].

A limitation of our study (Study II) where we used supervised learning to obtain classifiers that could distinguish between CAD and DCM samples was that the pool of samples was small (approximately 10 different individuals in each group), so it was not possible to use a subgroup of the samples as a test set for cross-validation. However, we used a "leave one out" algorithm for cross-validation to reduce overfitting. Future studies should ensure that a large population of samples is available, to facilitate the use of a stringent learning set/test set method. Admittedly, for some diseases and probably especially for cardiovascular research and diagnostics, this may be difficult to obtain. Possibly, in some cases, animal models can be used to explore molecular patterns that could classify different cardiovascular diseases or states of disease.

3.4 Gene annotation

3.4.1 Annotation databases

Microarray analysis generates lists of gene reporters that need to be connected to annotation data. To perform this individually for each gene is very time-consuming. Therefore, tools that allow batch searches have increased the usability of such tools. Tools for meta-analysis may be more useful and powerful if more types of data are integrated in a coherent way. Further, a dedicated annotation database that integrates various types of data from various sources is potentially better than any single database. However, not many such tools are available. The

main problems in building such an integrated database is difficulties in design and the need to update every time any one of its source databases is updated. This places a heavy burden on the team maintaining it. Given this, it is understandable that most tools available today use only one of the available annotation databases. In the future, at least in the context of the development of systems biology, databases that offer information from several sources will be essential.

However, there are still limitations even with the current annotation databases. First, the existing databases are incomplete. For virtually all sequenced organisms, only subsets of known genes are functionally annotated. Furthermore, most annotation databases are built by curators who manually review the existing literature. It is therefore possible that certain known facts might get temporarily overlooked. However, even more commonly, recent annotations are not in the databases yet because of the time lag necessary for the manual curation process. In this context we hope that our GO Annotation Tool [170](part of *GeneTools*, study III) can be a valuable tool. Until today most of the annotations have been performed by small groups of scientists, but now "regular" users can add annotations to their genes and choose to share these with the scientific community (export to GOA [211]). Hopefully, this can contribute to increasing the number of annotated genes and to speed up the annotation process.

A second problem with the annotation databases today is how genes are "named". Each resource often uses its own type of identifiers. For instance, GenBank uses accession numbers, UniGene uses cluster identifiers etc. Furthermore, genes are also represented by various company specific gene IDs. Various resources try to address this problem by maintaining different types of IDs together. For instance, beside its own gene names, EntrezGene database [163] also contains UniGene cluster IDs, and Affymetrix's NetAffx [169] provides RefSeq and GenBank accession numbers, beside its own array specific probe IDs. The burden of mapping various types of IDs to each other is left entirely to the researchers, who often have to revert to cutting and pasting lists of IDs from one database to another. The name space issue (connection between a specific sequence e.g. a probe and a gene) becomes crucial when trying to translate from lists of differentially expressed genes to functional profiles because the mapping from one type of identifier to another is not one to one. Consequently, the type of IDs used to specify the list of differentially regulated genes can potentially affect the results of the analysis [176,212], so the name space problem has yet to be solved. However, we recommend using primary IDs like e.g. GenBank accession numbers to decrease this problem.

Today, *GeneTools* is one of the largest sources that offer gene/protein annotations from several database sources. However, more annotations, through import of additional external databases should be added. A potential expansion may be to include information from Ensembl (genome browser) [213], the HapMap Project [214] and Kyoto Encyclopedia of Genes and Genomes (KEGG) [215]. To facilitate utilization of systems biology approaches, it would be advantageous if gene expression data were available. Finally, to ensure a good accessibility, the export formats can be extended e.g. by offering export in SBML (the systems biology markup language) [216] format.

3.4.2 Gene ontology

As mentioned earlier, the meta-analysis of microarray datasets largely depends on rapid access to previously described features/annotations of the genes being studied. Today, various publicly available resources exist that catalog various attributes of genes. However, most of this information is just text descriptions and not built on a common "language", so a common understanding of e.g. a description of a gene function or a pathway is difficult to find. However, some controlled vocabularies have been developed to address this problem. For example, GO represents a viable, and possibly long term, solution to the problem of inconsistent vocabulary. At least today GO is the most developed and used vocabulary for gene annotation, but other vocabularies like KEGG [215] are useful and has been popular, in the sense of analysing gene function.

Further, the current GO structure may not be the final answer. It is evident that there also are biological relationships between terms of the different subontologies (MF, BP and CC). A recent software named GO Annotation Toolbox (GOAT) [217], connects the three subontologies to enable GO to cover more biological knowledge, which also enables a more consistent use of GO, and therefore provides new opportunities for biological reasoning.

Most of the available GO tools use GO annotation data from a single public database. This has the advantage that the data is always as up to date as the database used. The disadvantage is that no single database offers a complete picture. For primary GO annotation data, the GO database (provided by the GO consortium [218]) is a comprehensive and up to date source since the contributing databases submit their data directly there. Other sources such as EntrezGene [163], derive their data from the GO database. In addition, EntrezGene curates these annotations, which in some situations may be useful. Our *GeneTools* database (Study III) obtains the GO annotations from EntrezGene, which we think is a good solution.

However, we also believe that using the GO database as the primary database can be a good solution if a "source" filter is available so that the users can choose which information from the GO database they want to use. A second problem is that certain pieces of information may also be imprecise or incorrect. For example, a large amount of the rat GO annotation associations are inferred exclusively from electronic annotations (i.e. without any expert human involvement). The vast majority of such electronic annotations are reasonably accurate. However, many such annotations are often made at a very high-level GO term which limits their usefulness. Furthermore, some of these inferences are incorrect. In our *e*GOn tool (Study III) we have implemented an "evidence" filter to solve this potential problem. To our knowledge, this is the only tool that offers this. A second potential solution can be to allow any type of weighting by quality of evidence in these GO tools, which is a limitation since experimentally derived annotations are more trustworthy than electronically inferred ones.

3.4.3 Gene-class testing

From microarray experiments, the investigators are often left with a large amount of unorganized findings. In response to the dual need to increase power to detect differential expression and to reduce the interpretive challenge that is posed by a long list of differentially expressed genes, gene-class testing has become a popular and widely accepted analytical tool. Gene classes are usually based on GO categories. Several gene-class testing methods and software packages are available, most of which use statistical tests to compare the number of genes in a class that are significant with the number that are expected under a particular null hypothesis. Several statistical tests for gene-class testing are offered in different GO tools. In our Study III (*GeneTools* [170]), we describe three different situations (master-target situation, mutually exclusive target-target situation and intersecting target-target situation), where different tests are used.

Tests for the master-target situation are offered in almost all tools and the Fisher's exact test is what most tools use. However, just a few tools offer tests for the mutually exclusive target-target situation, which we think is convenient when we want to look for differences between the representations of up- vs. down-regulated genes. In addition, to our knowledge we are the first to implement a test for the intersecting target-target situation. Such a test can be used to compare two gene reporter lists where a number of gene reporters are represented on both lists. In this way the intersecting target-target test can be used to investigate whether the GO

categories represented by these genes are over- or under-represented in the experiments behind the two lists.

Studies II and IV show how such tests can be used in different situations and how the results can be used in further hypothesis generation. However, a general cautionary remark on the statistical tests for association between two gene reporter lists is that they are based only on the gene lists submitted to the tool, and the raw data underlying the statistical analyses producing the gene reporter lists are not submitted to most tools. This means that e.g. *e*GOn does not offer permutation based methods for addressing the dependence structure between the genes. The statistical tests in *e*GOn are thus based on the assumption that under the null hypothesis the genes on the lists (or subsets of the lists in the intersecting target-target situation) act independently, as is also commonly assumed in other GO-tools.

A second important consideration when identifying statistically significant GO terms is the choice of the reference list of genes against which the P-values for each GO term in the results are calculated. Several tools (such as GOToolBox, GOstat, GoMiner, FatiGO, and GOTM) use the total set of genes in a genome as the reference or the total set of genes with GO annotations. Either of these may be an inappropriate choice when the input list of genes to these tools is a list of differentially expressed genes obtained from a microarray experiment, since the genes that are not present on a microarray do not have a chance of being selected as differentially regulated. The fundamental idea is to assign significance to various functional categories by comparing the observed number of genes in a specific category with the number of genes that might appear in the same category if a selection performed from the same pool were completely random. If the whole genome is considered as the reference, the pool considered when calculating the random choice includes all genes in the genome. At the same time, the pool available when actually selecting differentially regulated genes includes only the genes represented on the array used, since a gene that is not on the array can not be found to be differentially regulated. This represents an obvious contradiction of the assumptions of the statistical models used. Thus, in our GO tool *e*GOn, we use a master list (all reporters on array) as a reference list in the Master-Target test.

3.5 $^{86}Rb^{+}$-uptake

In Study I we used $^{86}Rb^{+}$ to measure potential K^{+} uptake through the H^{+}/K^{+}-ATPase in cardiac myocytes. A sample of the cell suspension, containing 10^{5} myocytes, was incubated at 37 C for 15 min (which was shown to be in the linear phase of $^{86}Rb^{+}$-uptake) with $^{86}Rb^{+}$. $^{86}Rb^{+}$-

uptake was measured in the absence and presence of Na^+/K^+-ATPase inhibitor ouabain and H^+/K^+-ATPase inhibitor SCH28080 which were added simultaneously with the $^{86}Rb^+$. Most H^+/K^+-ATPase inhibitor are just active in an acidic environment. However, in this experiment we chose to use SCH28080 because it is active at neutral pH.

In a control experiment the interaction of ouabain and SCH28080 on the $^{86}Rb^+$-uptake was tested at concentrations giving submaximal inhibitions both separately and combined. We showed that the selective Na^+/K^+-ATPase inhibitor ouabain maximally reduced the $^{86}Rb^+$-uptake by about 70%, which agreed with previous results by Viko et al. [219]. In addition, we found that the H^+/K^+-ATPase specific blocker SCH28080 reduced $^{86}Rb^+$-uptake by up to 70%. These observations suggest that at high concentrations, these agents directly or indirectly inhibit some common uptake mechanisms. At 1.0 X 10^{-4} M, however, SCH28080 did not interfere significantly with the ^{3}H-ouabain binding to the Na^+/K^+-ATPase, which is in contrast with the marked effect of unlabelled ouabain. Thus, at concentrations up to 1.0 X 10^{-4} M, SCH28080 seems to selectively inhibit the H^+/K^+-ATPase without significantly inhibiting the Na^+/K^+-ATPase. At this concentration, the $^{86}Rb^+$-uptake was reduced by about 25%, indicating that the H^+/K^+-ATPase may account for this fraction of the K^+-uptake. However, we tested the interaction between SCH28080 and ouabain at 1.0 X 10^{-4} and 0.5 X 10^{-4} M, respectively, in order to minimize interaction of these two agents on $^{86}Rb^+$-uptake mechanisms. Their inhibitory effects on the $^{86}Rb^+$-uptake were almost exactly additive, indicating that at these concentrations SCH28080 and ouabain act on different uptake mechanisms. Thus these results are compatible with a contribution from the H^+/K^+-ATPase to the total $^{86}Rb^+$-uptake. However, an accurate quantitative estimation of this contribution would depend upon a more exact knowledge of the selectivity of SCH28080 for the H^+/K^+-ATPase vs. the Na^+/K^+-ATPase than we obtained in this study.

4. Summary of results

Study I

- H^+/K^+-ATPase gene and protein expression was detected in heart tissue and localized to cardiac myocytes, by sequencing, rt-PCR, Western blotting, and immuno-cytochemistry.
- Competitive rt-PCR analysis indicated a significant up-regulation of the myocardial H^+/K^+-ATPase in heart failure after myocardial infarction, and the level of gene expression was related to infarction size.
- Both ouabain and the selective H^+/K^+-ATPase inhibitor Schering 28080 reduced $^{86}Rb^+$-uptake at maximum specific inhibition, by 70 and 25%, respectively; the effects were additive.

Study II

- We identified differential expression of 153 and 147 genes, respectively, in coronary artery disease (CAD) or dilated cardiomyopathy (DCM) versus non-failing hearts.
- Gene-class testing analysis of gene ontology (GO) biological process annotations indicated aetiology-specific patterns between CAD and DCM, primarily related to genes involved in catabolism and in regulation of protein kinase activity.
- Gene expression classifiers were developed and used for class prediction of random samples of CAD and DCM hearts. Most confident discrimination was obtained when the classifier included up to seven genes with the highest *t*-statistics (corresponding to $p<0.001$).
- The best classifier based on the AUC (0.89) measures correctly predicted group assignment in 16 of 20 samples, resulting in a test sensitivity of 0.750 and a specificity of 0.833.
- The best classifiers frequently included Matrix metalloproteinase 3, Fibulin 1, ATP-binding cassette, sub-family B member 1, and Iroquois homeobox protein 5.

Study III

- *GeneTools* is a web-service, providing access to a database that brings together information from a broad range of resources of gene information.
- *GeneTools* provides three different tools:

I. *NMC Annotation Tool*, which offers annotations from several databases like UniGene, Entrez Gene, SwissProt and GeneOntology, in both single- and batch search mode.

II. *GO Annotator Tool*, where users can add new gene ontology (GO) annotations to genes of interest. Such user-defined GO annotations can be used in further analysis or exported for public distribution.

III. *e*GOn, which is a tool for visualization and statistical hypothesis testing of GO category representation.

- As the first GO tool, *e*GOn supports hypothesis testing for three different situations (Master-Target situation, Mutually Exclusive Target-Target situation, and Intersecting Target-Target situation).
- An important additional function is an evidence-code filter that allows users to select the GO annotations for the analysis.

Study IV

- At all time points, three genes; Natriuretic peptide-precursor A, Interferon regulatory factor 3 (Irf3), and D-serine modulator-1, were up-regulated in CHF, and one gene, RAD23a homolog, in response to exercise.
- Irf3 may be a potential drug target, since it is likely to mediate the effects of peroxisome proliferator-activated receptors, which control fatty-acid oxidation in cardiac hypertrophy.
- Genes involved in fatty-acid metabolism were down-regulated in CHF, but not in response to exercise.
- Physiological hypertrophy was associated with less comprehensive changes in gene expression, indicating that post-transcriptional and post-translational regulation may be more important than in pathological hypertrophy.

5. Results and discussion

5.1 Functional genomics in cardiovascular research

Since human cardiac tissue is less accessible than e.g. tumor material, expression profiling has been performed to a lesser extent in human cardiac disease. The first cardiac expression profiles were based on analyses of expressed sequence tags (ESTs) obtained from cDNA libraries, leading to catalogues of genes expressed in normal or hypertrophied hearts [220-222]. These studies were followed by microarray analyses identifying genes (gene discovery) with differential expression levels in failing hearts. More recently, attempts were conducted to classify groups (supervised or unsupervised methods) of patients with end-stage heart failure based on their expression profile. In relation to all this, our Studies II and IV are natural further developments of what is previously done in this field of cardiovascular functional genomics.

5.2 Heart failure and gene expression

Researchers have long been interested in the development, progression and treatment of HF. Mouse and rat models of HF and cardiovascular disease have been created to enable studies of the disease mechanisms, devices to assist the heart in its pumping activity have been designed, and drugs to boost heart function have been developed. However, as of yet, the underlying mechanisms of heart disease remain uncertain and the therapies to treat it are still imperfect. Seeking to gain a better understanding of HF and find new ways to diagnose and treat it, we (by the studies in this thesis) and several others have examined the activity of genes in failing hearts. We aimed at studying both the causes and effects of heart failure, and one of the ways to do that is to look at changes in gene expression. For example, in Study I, we used standard rt-PCR and competitive rt-PCR to detect regulation of expression of H^+/K^+ in relation to heart infarction and during development. However, the more recent development of the DNA microarray technology enabled us and others to simultaneously study gene expression of tens of thousands of gene at once. One of our main aims in Study II and IV was to look for gene expression patterns in “normal” hearts versus diseased hearts, compare “acute” versus “chronic” phase and look for aetiology specific differences.

Like in most other studies we found that a set of genes and the molecular functions that they contribute to are regulated in the same way in different forms of HF diseases, reflecting a reaction to the pathological stimuli. For example, a high proportion of the differentially

expressed genes in the HF samples we measured in Study II and IV were related to e.g. cell communication, signal transduction, metabolism, transcription and development. Among the genes differentially expressed in HF we found an over representation of differentially expressed genes related to e.g. response to stress, defense response and inflammatory response, indicating that these processes are important in defining the HF specific response. Several other reports have shown similar patterns. For example, Yang et al. [223] was one of the first to publish gene discovery results from microarray data. They used high-density oligonucleotide arrays to explore changes in expression of ~7000 genes in 2 non failing and 2 failing human hearts with diagnoses of end-stage ischemic and dilated cardiomyopathy, respectively. They reported altered expression of cytoskeletal and myofibrillar genes, genes responsible for degradation and disassembly of myocardial proteins, genes involved in metabolism, genes responsible for protein synthesis, and genes encoding stress proteins. Like in this work, many authors have published "lists" of genes with altered expression in HF of e.g. CAD and DCM [37,224-230], where most studies involved small sample sizes and binary comparisons such as failing and non failing hearts. In addition, several papers have described the same global gene expression pattern in rodent models of HF [29,231-234]. Common for all these and our studies of HF, is an observed general change in expression levels of genes encoding cytoskeletal and extracellular matrix (ECM) proteins, as well as proteins involved in apoptosis and intracellular signaling, energy and lipid metabolism, cell communication and calcium-handling pathways. Together these studies, using genome wide gene expression arrays and making "lists" of differentially expressed genes which is a relatively easy but "rough" method to create general hypotheses, provided insights into novel genetic pathways and therapeutic targets. This may serve as the basis for studies involving molecular signature analysis. However, we believe that more sophisticated data analysis methods will be used to extract information from microarray data in the future. Such methods may be classification (e.g. supervised learning or PCA), connection to functional information (e.g. GO or KEGG), and modeling by systems biology approaches. The extended use of GO information in gene-class testing using statistical hypothesis testing has been very popular, but not many groups working with cardiovascular systems have published work using such methods and few attempts have been made to characterize genome-wide differences in gene expression patterns between e.g. CAD and DCM hearts**.** In our Study II and IV we extensively used such methods and this will be discussed in the next chapters.

5.2.1 Pathological and physiological hypertrophy signals

Cardiac hypertrophy responses can be found both in a physiological and pathologic setting, but the hypertrophy responses are not homogenous as shown in Study IV. Whereas physiological hypertrophy is characterized by eccentric growth, pathologic remodeling may be of either eccentric or concentric nature, depending on whether it is in response to pressure or volume overload [22]. Although the pathologic hypertrophy process is initially compensatory to an increased workload, it frequently progresses into detrimental remodeling and cardiac dysfunction, heart failure, arrhythmia and sudden death [235]. For example, in our MI model (Study IV) we measured that LV weight had increased by 45% at day 7 ($p<0.01$), and further to 53% ($p<0.01$) after 92 days, compared to sham animals. In addition previous studies in our laboratory have shown increased diastolic LV diameter, LV pressure and reduced peak dP/dt, peak –dP/dt and peak-systolic pressures already one week after coronary artery ligation. Together, this indicates a hypertrophy response already after 1 week [75,177,178]. On the other hand, cardiac hypertrophy induced by exercise training is considered a favorable adaptive response of the heart to regular physical activity and increase in bodily demands [236], and is characterized by increases in left ventricular (LV) chamber size, wall thickness and mass [237]. Several studies in our laboratory have demonstrated that the described exercise program elicits cardiac hypertrophy, both at the organ and cellular level [185,187,188,238,239]. LV weights increased by ~10% and 20-30%, whereas LV cardiac myocyte lengths increased ~6% and 10-15%, after 4 and 8 weeks of exercise, respectively, while a single exercise bout does not elicit any detectable increase in LV mass or myocyte size. Moreover, the cardiac growth in this exercise model is also associated with improved contractile function and Ca^{2+} handling by the cardiac myocytes [187,188,238,239]. Thus, it is an established, well verified and good model to study physiological hypertrophy of the heart.

The fact that physiological hypertrophy does not associate with dysfunction, but rather with sustained or improved function, leads to the question of what differentiates physiological from pathologic cardiac hypertrophy. It has been suggested that stimuli-specific hypertrophy responses may be associated with distinct molecular signatures [240,241].

In Study IV, we compared gene expression pattern from physiological (exercise training) and pathological (MI) hypertrophy models. One of the striking results was that a significantly lower number of differentially expressed genes was found in response to physiological compared to pathological hypertrophy. In addition there were no large differences in the numbers of differentially expressed genes during the studied time period in response to

physiological hypertrophy as compared to pathological hypertrophy where a significantly higher number of genes were differentially expressed in the acute phase vs. the chronic phase. This corresponds with other studies [29] and may be explained by the physiological stimulus being weaker than the pathological stimulus. Moreover, with the fact that relatively few genes are differentially expressed in response to physiological hypertrophy, our results (Study IV) indicate that post transcriptional processes, including translation regulation are important in development of physiological hypertrophy. Recently, it has been shown that activation of the IGF-1/ Phosphoinositide 3-kinase/Akt pathway is important for induction of physiologic, but not pathologic hypertrophy of the heart. The action of this pathway is mainly to increase ribosomal activity and protein synthesis without inducing a greater expression of hypertrophy-related genes; thus, improving translational efficiency [63]. Moreover, our observation in Study IV that genes related to protein amino acid dephosphorylation were over represented among the differentially expressed genes in response to physiological hypertrophy, supports the hypothesis that other factors than mRNA abundance may be important.

In Study IV the hypertrophy marker gene Natriuretic peptide precursor A (Nppa/ANF) was found to be up-regulated at all time points in response to MI. This correlates well with previous data obtained in our lab from the same rat MI model [177] using competitive rt-PCR for detection of gene expression. We found the hypertrophy marker Nppa/ANF to be up-regulated at the first two time points (1 and 4 hours after a single exercise bout), but not later in the hypertrophy process. Increased mRNA Nppa/ANF levels have also been found in response to some "chronic" training models, for example rats subjected to swimming [242,243], dogs subjected to a treadmill training program [244], and mice subjected to a wheel running program [245]. However, similarly to our study, in rats subjected to weeks of regular treadmill running [244,246,247] no induction of mRNA Nppa/ANF levels were found. Together, these results indicate that increased ANF expression is mediated at the transcriptional level as an early response to exercise and therefore the main signal may be the acute mechanical stretching of cardiac myocytes and not the hypertrophy itself.

To further identify biological processes differently represented between physiological and pathological hypertrophy, we looked for functional clusters among the differentially expressed genes, at different time points after MI and exercise training. The main finding was that genes related to fatty acid metabolism were down-regulated at all stages of MI, suggesting a switch in metabolism to carbohydrate metabolism. This was not seen in response to physiological hypertrophy. The switch in metabolism is a well known response to heart disease, however in

this study we show that it is a very early response to MI and that it continues during the whole disease process until HF is reached. It has been suggested that PPARs constitute the main control mechanism for myocardial metabolism by transcriptionally regulating genes encoding enzymes involved in fatty acid and glucose utilization [248], but a recent study [249] indicates a dissociation between gene and protein expression related to these processes. However, it is still a rationale for metabolic therapy to remedy cardiac hypertrophy and dysfunction in cardiac disease [250]. The possible role of the PPARs in the control of cardiac energy metabolism makes these regulatory pathways attractive targets for metabolic therapy. Interestingly, in addition to Nppa we found Interferon regulatory factor 3 (Irf3) to be overexpressed at all time points after MI (Study IV). Irf3 is a key player in the initial triggering of interferon gene transcription [251]. Moreover, it has been suggested that Irf3 may mediate the pro-apoptotic and anti-proliferative effects of peroxisome proliferator-activated receptors (PPARs) [252]. Little is still known about Irf3 in cardiac hypertrophy, but our results indicate a potentially important function. This should be further explored, and may be a subject for a follow up study.

5.2.2 Aetiology specific expression patterns

In our Study II we detected CAD and DCM aetiology-specific patterns by linking results from the gene expression analysis to GO annotations. First, for both groups of samples we found an over-representation of differentially expressed genes involved in cell communication, response to stress, response to external biotic stimulus, defense response, immune response and inflammatory response. However, some biological processes were over-represented in either CAD or in DCM and our main findings were that significantly more genes involved in catabolism were differentially expressed in CAD compared to DCM, which is consistent with catabolic/anabolic imbalance in CAD [253]. Moreover, a separate comparison of up-regulated and down-regulated genes was performed. A significantly higher number of genes involved in catabolism were up-regulated in CAD hearts compared to DCM hearts. Among 12 genes exclusively up-regulated in CAD, we found a sub-cluster of four genes involved in lipid catabolism, apolipoprotein C-II, (APOC2), lipoprotein lipase (LPL), phospholipase C, beta 1 (PLCB1) and phospholipase C, gamma 2 (PLCG2). In contrast, lipase, member H (LIPH) and phospholipase C, delta 1 (PLCD1) were down-regulated only in DCM. This indicates that lipid catabolism and lipid signaling is stimulated in end-stage CAD but not in DCM. This correlates with the results from Study IV, where we found an overrepresentation of genes

related to fatty acid metabolism (almost all down-regulated) in response to heart infarction, indicating a switch from lipid to carbohydrate metabolism.

During development of hypertrophy, phospholipase C (PLC) is increased, and in the failing heart PLC isozymes are decreased [254]. Normal, exercise-induced cardiac growth is mainly regulated by the growth hormone/IGF axis through the PI3K/Akt pathway. In contrast, pathological cardiac growth is triggered by autocrine and paracrine neurohormonal factors that signal through the Gq/phospholipase C pathway, leading to increased cytosolic calcium and PKC activation. In Study II we found IGF1 up-regulated in CAD, but not in DCM hearts, suggesting activation of a compensatory effect through the growth hormone/IGF pathway in end-stage CAD. However, this signal seems to be attenuated by an activation (up-regulation in both groups) of the phosphatase and tensin homolog (TPEN), which blocks signaling downstream of this pathway. Hence, our results indicate that the PLC pathway is activated in end-stage CAD, but not in DCM. These results are supported by the functional clustering, which shows that more genes regulating protein kinase activity are differentially expressed in DCM than CAD hearts. Among the genes regulating protein kinase activity that were differentially expressed in DCM but not in CAD, we found a sub-group of up-regulated genes, which inhibit protein kinase activity or cell division. Thus, an alternative to the Gq/phospholipase C pathway for hypertrophy may be active in end-stage DCM.

In this way Study II and IV have shown how it is possible to use gene-class testing methods and GO information to generate new hypotheses. Not many authors have yet described the use of such methods in cardiovascular research, but some good examples exist. For example, Barth et al. [255] used FatiGO [256] to look for regional gene expression patterns and significant associations of Gene Ontology terms within groups of genes expressed in left ventricular (LV) versus expression in right ventricular (RV) myocardium of human and mouse. Among other results they found that with respect to biological processes, functional groups related to cell-cell communication and response to external stimuli were more abundant in RV, while metabolic and energy-driving processes were over-represented in LV. Another example is a study published by Kong et al. [29] who used the ErmineJ software [257] to identify enriched GO classes in different rat models of physiological (exercise) and pathological (Dahl salt-sensitive rats) hypertrophy and heart failure. They found that during pathological hypertrophy, genes associated with the apoptosis pathway showed statistically significant over representation. In addition the authors suggested that gene clusters associated with glucose/insulin signaling, protein biosynthesis, and epidermal growth factor (EGF)

signaling pathways may be involved predominately during physiological hypertrophy. All together these results fit with what we found in Study II and IV.

5.2.3 Classification of heart disease using microarray data

In the past, supervised learning methods have mainly been used to predict cancer subtypes prognosis and treatment outcome [258-261], and just a few examples on heart failure are known. Kittleson et al. [262] were the first to use application of supervised learning analysis in cardiovascular disease. They identified a gene expression profile (best classifier included 90 genes) that differentiates the two major forms of cardiomyopathy, ischemic and non ischemic. A 16-sample training set was used to develop a prediction rule which was then tested in nine independent samples, including seven from a different institution. Their results showed that the aetiology signature performed perfectly in non ischemic samples (specificity 100%) but only identified one of three ischemic samples correctly (sensitivity 33%). In our Study II we obtained classifiers (consisting of 7 genes) using methods based on rough sets, to predict random samples of coronary artery diseased and dilated cardiomyopathic hearts. Like Kittelson et al. we were not able to predict all the samples to the correct group. Our best classifier predicted 16 of 20 samples correctly which gives a sensitivity of 0.750, a specificity of 0.833 and an AUC value of 0.89. This is not perfect and we believe the main reason for this is the limited number of samples. Another possible limitation is the biological variation between the samples in the groups, and the difficulty to obtain good quality RNA from human heart tissue samples. Similar studies on classification of cancer samples have given even better results [258,263,264], but in the some case of cancer much larger populations of samples are available and the samples may be more similar within each group.

However, taking this limitation in account, both studies indicate that microarray gene expression analysis may become an important complement to current standard methods, by providing more precise molecular diagnoses, and by sub-grouping existing disease categories into clusters with more accurate predictions of prognosis and response to treatment. For HF patients, the clinical benefit of expression profiling may not lie within classification at end-stage, but earlier in the course of the disease, where treatment strategies need to be improved. Further, microarray data may be incomplete and variable across platforms and between laboratories, if standardized protocols for hybridization and data acquisition are not used [193]. However, a central question is whether the predictive microarray data set can be used to extract a limited number of relevant genes whose expression levels convey the same

information as the whole set. Recently, Lossos et al. [264] evaluated a 6-gene-set QRT-PCR assay against original microarray data which predicted the clinical course of large-B-cell lymphoma and found that this 6-gene set was sufficient to predict survival. Thus, our assumptions for the future is that whole genome microarrays will be used to establish classifiers for prediction of e.g. disease or disease states, but for clinical use "focused" microarrays in e.g. microtiter plates, with just a limited numbers of probes, or a set of RT-PCR assays may be preferred.

In contrast to our approaches in Study II where the number of genes is reduced, unsupervised learning methods usually uses all data points for the experiments. The popularity of clustering and unsupervised analysis is also reflected in papers describing microarray experiments on HF, and recently some attempts have been made to characterize small groups of patients with end-stage HF based on hierarchical clustering of gene expression profiles. It is also possible to find attempts to use unsupervised methods to "classify" CAD and DCM. For example, Steenman et al. [229] were able to make distinct clusters of patients who were awaiting urgent cardiac transplantation, by hierarchical clustering analysis, but they were unable to identify specific clusters for the different aetiologies. A similar approach was used by Barrands et al. [224] where DCM hearts were successfully clustered against non failing hearts. Together these findings suggest that gene expression can be correlated to clinically relevant parameters in patients with HF. However, since the studies were focused on clustering (gene discovery), the observations could not be applied prospectively to identify and validate a gene expression signature with the intention to distinguish subjects based on relevant clinical parameters. This emphasizes the need for studies focused on molecular signature analysis. In contrast, our classifiers (Study II) selected a small number of genes whose expression levels distinguish between CAD and DCM, thus demonstrating, at least in this case, the feasibility of using microarray data and class prediction for studying HF pathobiology as compared to class discovery methods.

In relation to the results obtained in our Study II, a potentially clinically relevant follow-up study could be to classify different aetiologies of cardiomyopathy. This can help in the process of early diagnosis and correct selection of treatment. Another follow-up study may be to genotype different sub groups of cardiomyopathy patients with genome wide SNP microarray (arrays with up to 500K are now available). It is already known that genetic variation is involved in cardiomyopathy and arrhytmias [265,266].

5.3 H^+/K^+-ATPase activity and function in myocytes

During experiments on uptake of potassium and intracellular alkalization Falck et al. suggested that proton transport during hyperosmolal stress may be potassium dependent [267,268]. We thus anticipated that there might be another mechanism than the Na^+/K^+-ATPase and the $Na^+/K^+/2Cl^-$ co-transporter that could translocate K^+ into the cardiac myocytes. In previous studies, only Northern blot and cytochemical analysis had indicated presence of the gastric H^+/K^+-ATPase in rat myocardial tissue [269] and atrial myocytes [270,271], and there was no other report of similar findings at both RNA, protein and functional levels, in the left ventricle.

A study (Study I) to further investigate these indications was thus started. For the first time we have been able to show the presence of functional protein and gene expression of the gastric H^+/K^+-ATPase in rat heart and myocytes. We also showed regulation during development and in heart failure, indicating that the H^+/K^+-ATPase may play a crucial role in the mechanical and electrophysiological properties of the rat heart. Moreover and most importantly, we investigated whether the H^+/K^+-ATPase could account for a significant part of the $^{86}Rb^+$-uptake in cardiac ventricular myocytes. Our study suggests a contribution from the H^+/K^+-ATPase of about 25% of total $^{86}Rb^+$-uptake. Although its relative role cannot easily be translated into in vivo K^+-influxes, our data suggest that the gastric H^+/K^+-ATPase could contribute significantly to the regulation of myocardial K^+ and H^+ homeostasis. This may be particularly important in myocardial ischaemia and acidosis, provided that the H^+/K^+-ATPase activity is regulated as in vascular smooth muscle cells, where McCabe & Young [272] demonstrated that inhibitors of H^+/K^+-ATPase caused a rapid and marked decline in pHi and a marked depression in K^+ content. Their observations suggested a significant potential for maintenance of H^+ and K^+ gradients in vascular smooth muscle cells, which according to our results, also may be true in cardiac myocytes. However, in a recent publication, Yenisehiril et al. [273] investigated the effects of three specific H^+/K^+-ATPase inhibitors (Omeprazole, lansoprazole and SCH28080) on the contractile and chronotropic properties of isolated rat atrium. The possible changes in atrial action potential configuration were also studied. Yenisehiril et al. found that all three different H^+/K^+-ATPase inhibitors induced positive inotropic and negative chronotropic effects in the rat atria, in vitro. They also showed that these concentration dependent, reversible and reproducible effects seem not to be mediated by well known pathways that enhance contractility such as inhibition of Na^+/K^+-ATPase and

phosphodiesterase type III or activation of β-adrenoceptors. None of the pharmacological agents used in this study attenuated the cardiac effects of proton pump inhibitors. Therefore both they and we (Study I) suggest that H^+/K^+-ATPase accounts for K^+-uptake and has a possible role in intracellular pH regulation of cardiomyocytes. However, intracellular acidification is known to reduce force of contraction in cardiac muscle cells [274] and if intracellular acidosis reduces force of contraction, inhibition of H^+/K^+-ATPase is expected to decrease the amplitude of atrial contractions.

In our study (Study I) we demonstrated that SCH 28080 did not interfere significantly with the ^{3}H-ouabain binding to the Na^+/K^+-ATPase in rat cardiomyocytes and we concluded that SCH 28080 and ouabain act on different $^{86}Rb^+$-uptake mechanisms. These observations are compatible with the recent findings by Yenisehiril et al. [273]. They found that the cardiac effects of proton pump inhibitors operate through a mechanism different from Na^+/K^+-ATPase inhibition and they conclude that compounds known to inhibit H^+/K^+-ATPase increased contractility, decreased heart rate and exerted antiarrhythmic actions in isolated rat atria. Yenisehiril et al. also suggested that these effects appeared to be mediated by inhibition of myocardial H^+/K^+-ATPase and seem to be a result of prolongation of action potential duration. Moreover, a recent publication by Kemi et al. [86] concludes that H^+/K^+-ATPase does not contribute significantly to pHi maintenance. However, the complete role of the H^+/K^+-ATPase in pH-regulation and/or cell volume regulation has not yet been completely determined, and may be a subject to a potential follow up study.

5.4 Gene expression versus protein expression and molecular function

In our Studies II and IV, we have measured gene expression and made hypotheses of on the biological processes in the myocardial cells. However, it is not necessarily true that gene expression results in active protein. Translation of individual mRNA species into their encoded proteins is regulated at multiple levels: transcriptional control, RNA processing control, RNA transport control, translation control, mRNA degradation control and protein activity control, introducing possible discrepancies between mRNA and protein levels. As discussed earlier (regarding Study IV), this may be an important difference between the pathological and physiological hypertrophy response. However, as mRNA is eventually translated into protein, one might assume that there can be some sort of correlation between the level of mRNA and that of protein [275]. Attempts to correlate protein abundance with mRNA expression have had variable success, but several studies show a positive correlation in

the range from r = 0.48-0.76 [260,276]. This implies that there, at least in general, is a correlation between mRNA and protein abundance. In our Study I we show that gene expression and protein expression correlated at least for one gene and protein (H^+/K^+-ATPase). A wide assessment of protein function in experimental situations like this is, however, not possible with today's technical limitations. In the future proteomics may become more important and comparisons more informative, as larger datasets of both mRNA and proteomic measurements are obtained. It is also possible that other applications like tissue arrays and cell arrays (e.g. si RNA) will be available for the verification of hypotheses generated from genomic approaches and help to obtain a more complete understanding of how a cell works.

5.5 From genomes to systems

The present studies are examples of how research in functional genomics can be performed, switching between detailed functional studies of single genes/protein and high-throughput analysis and extensive use of bioinformatics tools. However, recent advances in the "omics" technologies, scientific computing, and mathematical modeling of biological processes will start to fundamentally impact the way we approach cardiovascular diseases and research. During the recent years we have witnessed the development of genome-scale functional screens, protein microarrays, databases and algorithms for "data-mining" and "text-mining". Altogether, this enables unprecedented descriptions of complex biological systems, which are testable by mathematical modeling and simulation. While the methods and tools are advancing, it is their iterative and combinatorial application that defines the systems biology approach [277], which now is the new "hype" in biology and medicine. Systems biology deals with understanding and depicting the complex and dynamic processes inside cells or organs. The wealth of data on different cell elements or functions, which were gathered at different levels of the life processes (genome, proteome, metabolome) may be put in a meaningful overall context and modelled on a computer, so that simulations and predictions may reduce the use of laboratory experiments. Through contextual understanding of the molecular mechanisms of disease, a systems approach may facilitate the identification and validation of therapeutic modulation of regulatory and metabolic networks, and hence help identify targets and biomarkers, as well as "off-target" effects and side effects of new drugs. In cardiovascular research, models of heart cells are becoming highly sophisticated and have benefited from four decades of interaction between experimental and simulation approaches [278]. Biological

simulation in cardiovascular research is now being applied over a wide range of pathways, cells and systems. The role of *in silico* biology in cardiovascular and pharmaceutical research is likely to become increasingly prominent as a result of the exploration of data generated through genomics and proteomic approaches.

6. Conclusions

- Gene expression classifiers could be used for class prediction of random clinical samples of CAD and DCM hearts. Best classifiers frequently included Matrix metalloproteinase 3, Fibulin 1, ATP-binding cassette, sub-family B member 1 and Iroquois homeobox protein 5.
- Analyzing gene expression at the level of gene ontology (GO) instead of at the level of single genes, detected the involvement of several biological processes e.g. transcription, metabolism, catabolism, muscle contraction, neurophysiological processes, defense response, and signal transduction, in myocardial hypertrophy and congestive heart failure.
- Gene-class testing analysis of GO biological process annotations indicated aetiology-specific patterns between CAD and DCM, primarily related to genes involved in catabolism and in regulation of protein kinase activity.
- A main difference in gene expression patterns between the models of pathological and physiological hypertrophy was the down-regulation of genes involved in fatty-acid metabolism in response to CHF, but not to exercise training.
- Physiological hypertrophy is associated with less comprehensive changes in gene expression than pathological hypertrophy, indicating that post-transcriptional and post-translational regulation may be more important.
- *GeneTools* is an “all in one” annotation tool, where the users can rapidly extract gene annotation data for thousands of genes or clones. *e*GOn includes tools for gene class comparison, that can perform hypothesis testing base on the representation of GO categories between gene reporter lists.
- H^+/K^+-ATPase is expressed in cardiac myocytes and accounts for 25% of the K^+-uptake in isolated ventricular cardiomyocytes, suggesting a role in the regulation of intracellular pH.

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58. Tarjei Rygnestad: DELIBERATE SELF-POISONING IN TRONDHEIM.
59. Arne Z. Henriksen: STUDIES ON CONSERVED ANTIGENIC DOMAINS ON MAJOR OUTER MEMBRANE PROTEINS FROM ENTEROBACTERIA.
60. Steinar Westin: UNEMPLOYMENT AND HEALTH: Medical and social consequences of a factory closure in a ten-year controlled follow-up study.
61. Ylva Sahlin: INJURY REGISTRATION, a tool for accident preventive work.
62. Helge Bjørnstad Pettersen: BIOSYNTHESIS OF COMPLEMENT BY HUMAN ALVEOLAR MACROPHAGES WITH SPECIAL REFERENCE TO SARCOIDOSIS.
63. Berit Schei: TRAPPED IN PAINFUL LOVE.
64. Lars J. Vatten: PROSPECTIVE STUDIES OF THE RISK OF BREAST CANCER IN A COHORT OF NORWEGIAN WOMAN.

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65. Kåre Bergh: APPLICATIONS OF ANTI-C5a SPECIFIC MONOCLONAL ANTIBODIES FOR THE ASSESSMENT OF COMPLEMENT ACTIVATION.
66. Svein Svenningsen: THE CLINICAL SIGNIFICANCE OF INCREASED FEMORAL ANTEVERSION.
67. Olbjørn Klepp: NONSEMINOMATOUS GERM CELL TESTIS CANCER: THERAPEUTIC OUTCOME AND PROGNOSTIC FACTORS.

68. Trond Sand: THE EFFECTS OF CLICK POLARITY ON BRAINSTEM AUDITORY EVOKED POTENTIALS AMPLITUDE, DISPERSION, AND LATENCY VARIABLES.
69. Kjetil B. Åsbakk: STUDIES OF A PROTEIN FROM PSORIATIC SCALE, PSO P27, WITH RESPECT TO ITS POTENTIAL ROLE IN IMMUNE REACTIONS IN PSORIASIS.
70. Arnulf Hestnes: STUDIES ON DOWN´S SYNDROME.
71. Randi Nygaard: LONG-TERM SURVIVAL IN CHILDHOOD LEUKEMIA.
72. Bjørn Hagen: THIO-TEPA.
73. Svein Anda: EVALUATION OF THE HIP JOINT BY COMPUTED TOMOGRAMPHY AND ULTRASONOGRAPHY.

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74. Martin Svartberg: AN INVESTIGATION OF PROCESS AND OUTCOME OF SHORT-TERM PSYCHODYNAMIC PSYCHOTHERAPY.
75. Stig Arild Slørdahl: AORTIC REGURGITATION.
76. Harold C Sexton: STUDIES RELATING TO THE TREATMENT OF SYMPTOMATIC NON-PSYCHOTIC PATIENTS.
77. Maurice B. Vincent: VASOACTIVE PEPTIDES IN THE OCULAR/FOREHEAD AREA.
78. Terje Johannessen: CONTROLLED TRIALS IN SINGLE SUBJECTS.
79. Turid Nilsen: PYROPHOSPHATE IN HEPATOCYTE IRON METABOLISM.
80. Olav Haraldseth: NMR SPECTROSCOPY OF CEREBRAL ISCHEMIA AND REPERFUSION IN RAT.
81. Eiliv Brenna: REGULATION OF FUNCTION AND GROWTH OF THE OXYNTIC MUCOSA.

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82. Gunnar Bovim: CERVICOGENIC HEADACHE.
83. Jarl Arne Kahn: ASSISTED PROCREATION.
84. Bjørn Naume: IMMUNOREGULATORY EFFECTS OF CYTOKINES ON NK CELLS.
85. Rune Wiseth: AORTIC VALVE REPLACEMENT.
86. Jie Ming Shen: BLOOD FLOW VELOCITY AND RESPIRATORY STUDIES.
87. Piotr Kruszewski: SUNCT SYNDROME WITH SPECIAL REFERENCE TO THE AUTONOMIC NERVOUS SYSTEM.
88. Mette Haase Moen: ENDOMETRIOSIS.
89. Anne Vik: VASCULAR GAS EMBOLISM DURING AIR INFUSION AND AFTER DECOMPRESSION IN PIGS.
90. Lars Jacob Stovner: THE CHIARI TYPE I MALFORMATION.
91. Kjell Å. Salvesen: ROUTINE ULTRASONOGRAPHY IN UTERO AND DEVELOPMENT IN CHILDHOOD.

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92. Nina-Beate Liabakk: DEVELOPMENT OF IMMUNOASSAYS FOR TNF AND ITS SOLUBLE RECEPTORS.
93. Sverre Helge Torp: *erb*B ONCOGENES IN HUMAN GLIOMAS AND MENINGIOMAS.
94. Olav M. Linaker: MENTAL RETARDATION AND PSYCHIATRY. Past and present.
95. Per Oscar Feet: INCREASED ANTIDEPRESSANT AND ANTIPANIC EFFECT IN COMBINED TREATMENT WITH DIXYRAZINE AND TRICYCLIC ANTIDEPRESSANTS.
96. Stein Olav Samstad: CROSS SECTIONAL FLOW VELOCITY PROFILES FROM TWO-DIMENSIONAL DOPPLER ULTRASOUND: Studies on early mitral blood flow.
97. Bjørn Backe: STUDIES IN ANTENATAL CARE.
98. Gerd Inger Ringdal: QUALITY OF LIFE IN CANCER PATIENTS.
99. Torvid Kiserud: THE DUCTUS VENOSUS IN THE HUMAN FETUS.
100. Hans E. Fjøsne: HORMONAL REGULATION OF PROSTATIC METABOLISM.
101. Eylert Brodtkorb: CLINICAL ASPECTS OF EPILEPSY IN THE MENTALLY RETARDED.
102. Roar Juul: PEPTIDERGIC MECHANISMS IN HUMAN SUBARACHNOID HEMORRHAGE.
103. Unni Syversen: CHROMOGRANIN A. Phsysiological and Clinical Role.

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104. Odd Gunnar Brakstad: THERMOSTABLE NUCLEASE AND THE *nuc* GENE IN THE DIAGNOSIS OF *Staphylococcus aureus* INFECTIONS.
105. Terje Engan: NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY OF PLASMA IN MALIGNANT DISEASE.
106. Kirsten Rasmussen: VIOLENCE IN THE MENTALLY DISORDERED.
107. Finn Egil Skjeldestad: INDUCED ABORTION: Timetrends and Determinants.
108. Roar Stenseth: THORACIC EPIDURAL ANALGESIA IN AORTOCORONARY BYPASS SURGERY.

109. Arild Faxvaag: STUDIES OF IMMUNE CELL FUNCTION *in mice infected with* MURINE RETROVIRUS.

1996

110. Svend Aakhus: NONINVASIVE COMPUTERIZED ASSESSMENT OF LEFT VENTRICULAR FUNCTION AND SYSTEMIC ARTERIAL PROPERTIES. Methodology and some clinical applications.
111. Klaus-Dieter Bolz: INTRAVASCULAR ULTRASONOGRAPHY.
112. Petter Aadahl: CARDIOVASCULAR EFFECTS OF THORACIC AORTIC CROSS-CLAMPING.
113. Sigurd Steinshamn: CYTOKINE MEDIATORS DURING GRANULOCYTOPENIC INFECTIONS.
114. Hans Stifoss-Hanssen: SEEKING MEANING OR HAPPINESS?
115. Anne Kvikstad: LIFE CHANGE EVENTS AND MARITAL STATUS IN RELATION TO RISK AND PROGNOSIS OF CANSER.
116. Torbjørn Grøntvedt: TREATMENT OF ACUTE AND CHRONIC ANTERIOR CRUCIATE LIGAMENT INJURIES. A clinical and biomechanical study.
117. Sigrid Hørven Wigers: CLINICAL STUDIES OF FIBROMYALGIA WITH FOCUS ON ETIOLOGY, TREATMENT AND OUTCOME.
118. Jan Schjøtt: MYOCARDIAL PROTECTION: Functional and Metabolic Characteristics of Two Endogenous Protective Principles.
119. Marit Martinussen: STUDIES OF INTESTINAL BLOOD FLOW AND ITS RELATION TO TRANSITIONAL CIRCULATORY ADAPATION IN NEWBORN INFANTS.
120. Tomm B. Müller: MAGNETIC RESONANCE IMAGING IN FOCAL CEREBRAL ISCHEMIA.
121. Rune Haaverstad: OEDEMA FORMATION OF THE LOWER EXTREMITIES.
122. Magne Børset: THE ROLE OF CYTOKINES IN MULTIPLE MYELOMA, WITH SPECIAL REFERENCE TO HEPATOCYTE GROWTH FACTOR.
123. Geir Smedslund: A THEORETICAL AND EMPIRICAL INVESTIGATION OF SMOKING, STRESS AND DISEASE: RESULTS FROM A POPULATION SURVEY.

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124. Torstein Vik: GROWTH, MORBIDITY, AND PSYCHOMOTOR DEVELOPMENT IN INFANTS WHO WERE GROWTH RETARDED *IN UTERO*.
125. Siri Forsmo: ASPECTS AND CONSEQUENCES OF OPPORTUNISTIC SCREENING FOR CERVICAL CANCER. Results based on data from three Norwegian counties.
126. Jon S. Skranes: CEREBRAL MRI AND NEURODEVELOPMENTAL OUTCOME IN VERY LOW BIRTH WEIGHT (VLBW) CHILDREN. A follow-up study of a geographically based year cohort of VLBW children at ages one and six years.
127. Knut Bjørnstad: COMPUTERIZED ECHOCARDIOGRAPHY FOR EVALUTION OF CORONARY ARTERY DISEASE.
128. Grethe Elisabeth Borchgrevink: DIAGNOSIS AND TREATMENT OF WHIPLASH/NECK SPRAIN INJURIES CAUSED BY CAR ACCIDENTS.
129. Tor Elsås: NEUROPEPTIDES AND NITRIC OXIDE SYNTHASE IN OCULAR AUTONOMIC AND SENSORY NERVES.
130. Rolf W. Gråwe: EPIDEMIOLOGICAL AND NEUROPSYCHOLOGICAL PERSPECTIVES ON SCHIZOPHRENIA.
131. Tonje Strømholm: CEREBRAL HAEMODYNAMICS DURING THORACIC AORTIC CROSSCLAMPING. An experimental study in pigs.

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132. Martinus Bråten: STUDIES ON SOME PROBLEMS REALTED TO INTRAMEDULLARY NAILING OF FEMORAL FRACTURES.
133. Ståle Nordgård: PROLIFERATIVE ACTIVITY AND DNA CONTENT AS PROGNOSTIC INDICATORS IN ADENOID CYSTIC CARCINOMA OF THE HEAD AND NECK.
134. Egil Lien: SOLUBLE RECEPTORS FOR **TNF** AND **LPS**: RELEASE PATTERN AND POSSIBLE SIGNIFICANCE IN DISEASE.
135. Marit Bjørgaas: HYPOGLYCAEMIA IN CHILDREN WITH DIABETES MELLITUS
136. Frank Skorpen: GENETIC AND FUNCTIONAL ANALYSES OF DNA REPAIR IN HUMAN CELLS.
137. Juan A. Pareja: SUNCT SYNDROME. ON THE CLINICAL PICTURE. ITS DISTINCTION FROM OTHER, SIMILAR HEADACHES.
138. Anders Angelsen: NEUROENDOCRINE CELLS IN HUMAN PROSTATIC CARCINOMAS AND THE PROSTATIC COMPLEX OF RAT, GUINEA PIG, CAT AND DOG.

139. Fabio Antonaci: CHRONIC PAROXYSMAL HEMICRANIA AND HEMICRANIA CONTINUA: TWO DIFFERENT ENTITIES?
140. Sven M. Carlsen: ENDOCRINE AND METABOLIC EFFECTS OF METFORMIN WITH SPECIAL EMPHASIS ON CARDIOVASCULAR RISK FACTORES.

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141. Terje A. Murberg: DEPRESSIVE SYMPTOMS AND COPING AMONG PATIENTS WITH CONGESTIVE HEART FAILURE.
142. Harm-Gerd Karl Blaas: THE EMBRYONIC EXAMINATION. Ultrasound studies on the development of the human embryo.
143. Noèmi Becser Andersen:THE CEPHALIC SENSORY NERVES IN UNILATERAL HEADACHES. Anatomical background and neurophysiological evaluation.
144. Eli-Janne Fiskerstrand: LASER TREATMENT OF PORT WINE STAINS. A study of the efficacy and limitations of the pulsed dye laser. Clinical and morfological analyses aimed at improving the therapeutic outcome.
145. Bård Kulseng: A STUDY OF ALGINATE CAPSULE PROPERTIES AND CYTOKINES IN RELATION TO INSULIN DEPENDENT DIABETES MELLITUS.
146. Terje Haug: STRUCTURE AND REGULATION OF THE HUMAN UNG GENE ENCODING URACIL-DNA GLYCOSYLASE.
147. Heidi Brurok: MANGANESE AND THE HEART. A Magic Metal with Diagnostic and Therapeutic Possibilites.
148. Agnes Kathrine Lie: DIAGNOSIS AND PREVALENCE OF HUMAN PAPILLOMAVIRUS INFECTION IN CERVICAL INTRAEPITELIAL NEOPLASIA. Relationship to Cell Cycle Regulatory Proteins and HLA DQBI Genes.
149. Ronald Mårvik: PHARMACOLOGICAL, PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STUDIES ON ISOLATED STOMACS.
150. Ketil Jarl Holen: THE ROLE OF ULTRASONOGRAPHY IN THE DIAGNOSIS AND TREATMENT OF HIP DYSPLASIA IN NEWBORNS.
151. Irene Hetlevik: THE ROLE OF CLINICAL GUIDELINES IN CARDIOVASCULAR RISK INTERVENTION IN GENERAL PRACTICE.
152. Katarina Tunòn: ULTRASOUND AND PREDICTION OF GESTATIONAL AGE.
153. Johannes Soma: INTERACTION BETWEEN THE LEFT VENTRICLE AND THE SYSTEMIC ARTERIES.
154. Arild Aamodt: DEVELOPMENT AND PRE-CLINICAL EVALUATION OF A CUSTOM-MADE FEMORAL STEM.
155. Agnar Tegnander: DIAGNOSIS AND FOLLOW-UP OF CHILDREN WITH SUSPECTED OR KNOWN HIP DYSPLASIA.
156. Bent Indredavik: STROKE UNIT TREATMENT: SHORT AND LONG-TERM EFFECTS
157. Jolanta Vanagaite Vingen: PHOTOPHOBIA AND PHONOPHOBIA IN PRIMARY HEADACHES

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158. Ola Dalsegg Sæther: PATHOPHYSIOLOGY DURING PROXIMAL AORTIC CROSS-CLAMPING CLINICAL AND EXPERIMENTAL STUDIES
159. xxxxxxxxx (blind number)
160. Christina Vogt Isaksen: PRENATAL ULTRASOUND AND POSTMORTEM FINDINGS – A TEN YEAR CORRELATIVE STUDY OF FETUSES AND INFANTS WITH DEVELOPMENTAL ANOMALIES.
161. Holger Seidel: HIGH-DOSE METHOTREXATE THERAPY IN CHILDREN WITH ACUTE LYMPHOCYTIC LEUKEMIA: DOSE, CONCENTRATION, AND EFFECT CONSIDERATIONS.
162. Stein Hallan: IMPLEMENTATION OF MODERN MEDICAL DECISION ANALYSIS INTO CLINICAL DIAGNOSIS AND TREATMENT.
163. Malcolm Sue-Chu: INVASIVE AND NON-INVASIVE STUDIES IN CROSS-COUNTRY SKIERS WITH ASTHMA-LIKE SYMPTOMS.
164. Ole-Lars Brekke: EFFECTS OF ANTIOXIDANTS AND FATTY ACIDS ON TUMOR NECROSIS FACTOR-INDUCED CYTOTOXICITY.
165. Jan Lundbom: AORTOCORONARY BYPASS SURGERY: CLINICAL ASPECTS, COST CONSIDERATIONS AND WORKING ABILITY.
166. John-Anker Zwart: LUMBAR NERVE ROOT COMPRESSION, BIOCHEMICAL AND NEUROPHYSIOLOGICAL ASPECTS.
167. Geir Falck: HYPEROSMOLALITY AND THE HEART.

168. Eirik Skogvoll: CARDIAC ARREST Incidence, Intervention and Outcome.
169. Dalius Bansevicius: SHOULDER-NECK REGION IN CERTAIN HEADACHES AND CHRONIC PAIN SYNDROMES.
170. Bettina Kinge: REFRACTIVE ERRORS AND BIOMETRIC CHANGES AMONG UNIVERSITY STUDENTS IN NORWAY.
171. Gunnar Qvigstad: CONSEQUENCES OF HYPERGASTRINEMIA IN MAN
172. Hanne Ellekjær: EPIDEMIOLOGICAL STUDIES OF STROKE IN A NORWEGIAN POPULATION. INCIDENCE, RISK FACTORS AND PROGNOSIS
173. Hilde Grimstad: VIOLENCE AGAINST WOMEN AND PREGNANCY OUTCOME.
174. Astrid Hjelde: SURFACE TENSION AND COMPLEMENT ACTIVATION: Factors influencing bubble formation and bubble effects after decompression.
175. Kjell A. Kvistad: MR IN BREAST CANCER – A CLINICAL STUDY.
176. Ivar Rossvoll: ELECTIVE ORTHOPAEDIC SURGERY IN A DEFINED POPULATION. Studies on demand, waiting time for treatment and incapacity for work.
177. Carina Seidel: PROGNOSTIC VALUE AND BIOLOGICAL EFFECTS OF HEPATOCYTE GROWTH FACTOR AND SYNDECAN-1 IN MULTIPLE MYELOMA.

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178. Alexander Wahba: THE INFLUENCE OF CARDIOPULMONARY BYPASS ON PLATELET FUNCTION AND BLOOD COAGULATION – DETERMINANTS AND CLINICAL CONSEQUENSES
179. Marcus Schmitt-Egenolf: THE RELEVANCE OF THE MAJOR hISTOCOMPATIBILITY COMPLEX FOR THE GENETICS OF PSORIASIS
180. Odrun Arna Gederaas: BIOLOGICAL MECHANISMS INVOLVED IN 5-AMINOLEVULINIC ACID BASED PHOTODYNAMIC THERAPY
181. Pål Richard Romundstad: CANCER INCIDENCE AMONG NORWEGIAN ALUMINIUM WORKERS
182. Henrik Hjorth-Hansen: NOVEL CYTOKINES IN GROWTH CONTROL AND BONE DISEASE OF MULTIPLE MYELOMA
183. Gunnar Morken: SEASONAL VARIATION OF HUMAN MOOD AND BEHAVIOUR
184. Bjørn Olav Haugen: MEASUREMENT OF CARDIAC OUTPUT AND STUDIES OF VELOCITY PROFILES IN AORTIC AND MITRAL FLOW USING TWO- AND THREE-DIMENSIONAL COLOUR FLOW IMAGING
185. Geir Bråthen: THE CLASSIFICATION AND CLINICAL DIAGNOSIS OF ALCOHOL-RELATED SEIZURES
186. Knut Ivar Aasarød: RENAL INVOLVEMENT IN INFLAMMATORY RHEUMATIC DISEASE. A Study of Renal Disease in Wegener's Granulomatosis and in Primary Sjögren's Syndrome
187. Trude Helen Flo: RESEPTORS INVOLVED IN CELL ACTIVATION BY DEFINED URONIC ACID POLYMERS AND BACTERIAL COMPONENTS
188. Bodil Kavli: HUMAN URACIL-DNA GLYCOSYLASES FROM THE UNG GENE: STRUCTRUAL BASIS FOR SUBSTRATE SPECIFICITY AND REPAIR
189. Liv Thommesen: MOLECULAR MECHANISMS INVOLVED IN TNF- AND GASTRIN-MEDIATED GENE REGULATION
190. Turid Lingaas Holmen: SMOKING AND HEALTH IN ADOLESCENCE; THE NORD-TRØNDELAG HEALTH STUDY, 1995-97
191. Øyvind Hjertner: MULTIPLE MYELOMA: INTERACTIONS BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MICROENVIRONMENT
192. Asbjørn Støylen: STRAIN RATE IMAGING OF THE LEFT VENTRICLE BY ULTRASOUND. FEASIBILITY, CLINICAL VALIDATION AND PHYSIOLOGICAL ASPECTS
193. Kristian Midthjell: DIABETES IN ADULTS IN NORD-TRØNDELAG. PUBLIC HEALTH ASPECTS OF DIABETES MELLITUS IN A LARGE, NON-SELECTED NORWEGIAN POPULATION.
194. Guanglin Cui: FUNCTIONAL ASPECTS OF THE ECL CELL IN RODENTS
195. Ulrik Wisløff: CARDIAC EFFECTS OF AEROBIC ENDURANCE TRAINING: HYPERTROPHY, CONTRACTILITY AND CALCUIM HANDLING IN NORMAL AND FAILING HEART
196. Øyvind Halaas: MECHANISMS OF IMMUNOMODULATION AND CELL-MEDIATED CYTOTOXICITY INDUCED BY BACTERIAL PRODUCTS
197. Tore Amundsen: PERFUSION MR IMAGING IN THE DIAGNOSIS OF PULMONARY EMBOLISM

198. Nanna Kurtze: THE SIGNIFICANCE OF ANXIETY AND DEPRESSION IN FATIQUE AND PATTERNS OF PAIN AMONG INDIVIDUALS DIAGNOSED WITH FIBROMYALGIA: RELATIONS WITH QUALITY OF LIFE, FUNCTIONAL DISABILITY, LIFESTYLE, EMPLOYMENT STATUS, CO-MORBIDITY AND GENDER
199. Tom Ivar Lund Nilsen: PROSPECTIVE STUDIES OF CANCER RISK IN NORD-TRØNDELAG: THE HUNT STUDY. Associations with anthropometric, socioeconomic, and lifestyle risk factors
200. Asta Kristine Håberg: A NEW APPROACH TO THE STUDY OF MIDDLE CEREBRAL ARTERY OCCLUSION IN THE RAT USING MAGNETIC RESONANCE TECHNIQUES

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201. Knut Jørgen Arntzen: PREGNANCY AND CYTOKINES
202. Henrik Døllner: INFLAMMATORY MEDIATORS IN PERINATAL INFECTIONS
203. Asta Bye: LOW FAT, LOW LACTOSE DIET USED AS PROPHYLACTIC TREATMENT OF ACUTE INTESTINAL REACTIONS DURING PELVIC RADIOTHERAPY. A PROSPECTIVE RANDOMISED STUDY.
204. Sylvester Moyo: STUDIES ON STREPTOCOCCUS AGALACTIAE (GROUP B STREPTOCOCCUS) SURFACE-ANCHORED MARKERS WITH EMPHASIS ON STRAINS AND HUMAN SERA FROM ZIMBABWE.
205. Knut Hagen: HEAD-HUNT: THE EPIDEMIOLOGY OF HEADACHE IN NORD-TRØNDELAG
206. Li Lixin: ON THE REGULATION AND ROLE OF UNCOUPLING PROTEIN-2 IN INSULIN PRODUCING ß-CELLS
207. Anne Hildur Henriksen: SYMPTOMS OF ALLERGY AND ASTHMA VERSUS MARKERS OF LOWER AIRWAY INFLAMMATION AMONG ADOLESCENTS
208. Egil Andreas Fors: NON-MALIGNANT PAIN IN RELATION TO PSYCHOLOGICAL AND ENVIRONTENTAL FACTORS. EXPERIENTAL AND CLINICAL STUDES OF PAIN WITH FOCUS ON FIBROMYALGIA
209. Pål Klepstad: MORPHINE FOR CANCER PAIN
210. Ingunn Bakke: MECHANISMS AND CONSEQUENCES OF PEROXISOME PROLIFERATOR-INDUCED HYPERFUNCTION OF THE RAT GASTRIN PRODUCING CELL
211. Ingrid Susann Gribbestad: MAGNETIC RESONANCE IMAGING AND SPECTROSCOPY OF BREAST CANCER
212. Rønnaug Astri Ødegård: PREECLAMPSIA – MATERNAL RISK FACTORS AND FETAL GROWTH
213. Johan Haux: STUDIES ON CYTOTOXICITY INDUCED BY HUMAN NATURAL KILLER CELLS AND DIGITOXIN
214. Turid Suzanne Berg-Nielsen: PARENTING PRACTICES AND MENTALLY DISORDERED ADOLESCENTS
215. Astrid Rydning: BLOOD FLOW AS A PROTECTIVE FACTOR FOR THE STOMACH MUCOSA. AN EXPERIMENTAL STUDY ON THE ROLE OF MAST CELLS AND SENSORY AFFERENT NEURONS

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216. Jan Pål Loennechen: HEART FAILURE AFTER MYOCARDIAL INFARCTION. Regional Differences, Myocyte Function, Gene Expression, and Response to Cariporide, Losartan, and Exercise Training.
217. Elisabeth Qvigstad: EFFECTS OF FATTY ACIDS AND OVER-STIMULATION ON INSULIN SECRETION IN MAN
218. Arne Åsberg: EPIDEMIOLOGICAL STUDIES IN HEREDITARY HEMOCHROMATOSIS: PREVALENCE, MORBIDITY AND BENEFIT OF SCREENING.
219. Johan Fredrik Skomsvoll: REPRODUCTIVE OUTCOME IN WOMEN WITH RHEUMATIC DISEASE. A population registry based study of the effects of inflammatory rheumatic disease and connective tissue disease on reproductive outcome in Norwegian women in 1967-1995.
220. Siv Mørkved: URINARY INCONTINENCE DURING PREGNANCY AND AFTER DELIVERY: EFFECT OF PELVIC FLOOR MUSCLE TRAINING IN PREVENTION AND TREATMENT
221. Marit S. Jordhøy: THE IMPACT OF COMPREHENSIVE PALLIATIVE CARE
222. Tom Christian Martinsen: HYPERGASTRINEMIA AND HYPOACIDITY IN RODENTS – CAUSES AND CONSEQUENCES
223. Solveig Tingulstad: CENTRALIZATION OF PRIMARY SURGERY FOR OVARAIN CANCER. FEASIBILITY AND IMPACT ON SURVIVAL

224. Haytham Eloqayli: METABOLIC CHANGES IN THE BRAIN CAUSED BY EPILEPTIC SEIZURES
225. Torunn Bruland: STUDIES OF EARLY RETROVIRUS-HOST INTERACTIONS – VIRAL DETERMINANTS FOR PATHOGENESIS AND THE INFLUENCE OF SEX ON THE SUSCEPTIBILITY TO FRIEND MURINE LEUKAEMIA VIRUS INFECTION
226. Torstein Hole: DOPPLER ECHOCARDIOGRAPHIC EVALUATION OF LEFT VENTRICULAR FUNCTION IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION
227. Vibeke Nossum: THE EFFECT OF VASCULAR BUBBLES ON ENDOTHELIAL FUNCTION
228. Sigurd Fasting: ROUTINE BASED RECORDING OF ADVERSE EVENTS DURING ANAESTHESIA – APPLICATION IN QUALITY IMPROVEMENT AND SAFETY
229. Solfrid Romundstad: EPIDEMIOLOGICAL STUDIES OF MICROALBUMINURIA. THE NORD-TRØNDELAG HEALTH STUDY 1995-97 (HUNT 2)
230. Geir Torheim: PROCESSING OF DYNAMIC DATA SETS IN MAGNETIC RESONANCE IMAGING
231. Catrine Ahlén: SKIN INFECTIONS IN OCCUPATIONAL SATURATION DIVERS IN THE NORTH SEA AND THE IMPACT OF THE ENVIRONMENT
232. Arnulf Langhammer: RESPIRATORY SYMPTOMS, LUNG FUNCTION AND BONE MINERAL DENSITY IN A COMPREHENSIVE POPULATION SURVEY. THE NORD-TRØNDELAG HEALTH STUDY 1995-97. THE BRONCHIAL OBSTRUCTION IN NORD-TRØNDELAG STUDY
233. Einar Kjelsås: EATING DISORDERS AND PHYSICAL ACTIVITY IN NON-CLINICAL SAMPLES
234. Arne Wibe: RECTAL CANCER TREATMENT IN NORWAY – STANDARDISATION OF SURGERY AND QUALITY ASSURANCE

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235. Eivind Witsø: BONE GRAFT AS AN ANTIBIOTIC CARRIER
236. Anne Mari Sund: DEVELOPMENT OF DEPRESSIVE SYMPTOMS IN EARLY ADOLESCENCE
237. Hallvard Lærum: EVALUATION OF ELECTRONIC MEDICAL RECORDS – A CLINICAL TASK PERSPECTIVE
238. Gustav Mikkelsen: ACCESSIBILITY OF INFORMATION IN ELECTRONIC PATIENT RECORDS; AN EVALUATION OF THE ROLE OF DATA QUALITY
239. Steinar Krokstad: SOCIOECONOMIC INEQUALITIES IN HEALTH AND DISABILITY. SOCIAL EPIDEMIOLOGY IN THE NORD-TRØNDELAG HEALTH STUDY (HUNT), NORWAY
240. Arne Kristian Myhre: NORMAL VARIATION IN ANOGENITAL ANATOMY AND MICROBIOLOGY IN NON-ABUSED PRESCHOOL CHILDREN
241. Ingunn Dybedal: NEGATIVE REGULATORS OF HEMATOPOIETEC STEM AND PROGENITOR CELLS
242. Beate Sitter: TISSUE CHARACTERIZATION BY HIGH RESOLUTION MAGIC ANGLE SPINNING MR SPECTROSCOPY
243. Per Arne Aas: MACROMOLECULAR MAINTENANCE IN HUMAN CELLS – REPAIR OF URACIL IN DNA AND METHYLATIONS IN DNA AND RNA
244. Anna Bofin: FINE NEEDLE ASPIRATION CYTOLOGY IN THE PRIMARY INVESTIGATION OF BREAST TUMOURS AND IN THE DETERMINATION OF TREATMENT STRATEGIES
245. Jim Aage Nøttestad: DEINSTITUTIONALIZATION AND MENTAL HEALTH CHANGES AMONG PEOPLE WITH MENTAL RETARDATION
246. Reidar Fossmark: GASTRIC CANCER IN JAPANESE COTTON RATS
247. Wibeke Nordhøy: MANGANESE AND THE HEART, INTRACELLULAR MR RELAXATION AND WATER EXCHANGE ACROSS THE CARDIAC CELL MEMBRANE

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248. Sturla Molden: QUANTITATIVE ANALYSES OF SINGLE UNITS RECORDED FROM THE HIPPOCAMPUS AND ENTORHINAL CORTEX OF BEHAVING RATS
249. Wenche Brenne Drøyvold: EPIDEMIOLOGICAL STUDIES ON WEIGHT CHANGE AND HEALTH IN A LARGE POPULATION. THE NORD-TRØNDELAG HEALTH STUDY (HUNT)
250. Ragnhild Støen: ENDOTHELIUM-DEPENDENT VASODILATION IN THE FEMORAL ARTERY OF DEVELOPING PIGLETS

251. Aslak Steinsbekk: HOMEOPATHY IN THE PREVENTION OF UPPER RESPIRATORY TRACT INFECTIONS IN CHILDREN
252. Hill-Aina Steffenach: MEMORY IN HIPPOCAMPAL AND CORTICO-HIPPOCAMPAL CIRCUITS
253. Eystein Stordal: ASPECTS OF THE EPIDEMIOLOGY OF DEPRESSIONS BASED ON SELF-RATING IN A LARGE GENERAL HEALTH STUDY (THE HUNT-2 STUDY)
254. Viggo Pettersen: FROM MUSCLES TO SINGING: THE ACTIVITY OF ACCESSORY BREATHING MUSCLES AND THORAX MOVEMENT IN CLASSICAL SINGING
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