IMPAIRED TISSUE RESPONSIVENESS TO B-TYPE NATRIURETIC PEPTIDE IN HEART FAILURE: BIOCHEMICAL BASES

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Abstract

Release of the B-type natriuretic peptide (BNP) is increased in heart failure (HF). Physiologically, BNP exerts natriuretic, diuretic, vasodilator and cardioprotective effects. A number of clinical investigations carried out with synthetic BNP for the treatment of HF have suggested that BNP-based restoration of homeostasis is inadequate. This equivocal clinical benefit of a recombinant BNP in HF raises the possibility of attenuated BNP response.

The objective of this thesis is an examination of three aspects of BNP-related cardiovascular pathophysiology. The first issue tested was the effect of BNP in isolated neutrophils of control subjects via neutrophil superoxide (O_2^-) generation. The second issue was integrity of BNP effects in acute and chronic HF patients, and the third was maintenance of BNP effect in the acute phase of Tako-tsubo cardiomyopathy (TTC), a form of "stress-induced" cardiomyopathy and another condition associated with increased BNP release.

The study utilized the technique of electron paramagnetic resonance spectroscopy to quantitate the extent of O_2^- generation from isolated neutrophils. In control subjects, the data showed significant suppression of O_2^- generation in PMA- and fMLP-stimulated neutrophils. This effect was not affected by either age or gender of the study population. The effect of BNP was mimicked by a cell-permeable cGMP analog (8-pCPT-cGMP) and partially restored by a protein kinase G (PKG) inhibitor KT5823. Furthermore, BNP inhibited the fMLP-induced phosphorylation of the NAD(P)H oxidase subunit p47phox at ser345. These findings led to the conclusion that BNP suppression of O_2^- generation is mediated by the cGMP-PKG signaling pathway.

The studies concerning HF patients had two major components: (a) examination of the BNP effect in acute HF patients and (b) determination of changes in effect with chronic treatment of such patients.

Studies with acute HF patients showed a significant attenuation of BNP effects on suppressing neutrophil O_2^- generation compared with control subjects. However, 8-p-CPT-cGMP effects were retained, which indicates that BNP effects were attenuated at the level of natriuretic peptide receptor level. Furthermore, the effect of BNP on inhibition of the fMLP-induced phosphorylation of p47phox at ser345 was lost in acute HF patients. Comparison of the acute and chronic HF patients revealed a partial restoration of BNP effects, especially in younger patients.

TTC is associated with acute release of BNP into plasma as a result of inflammatory change in the heart. It was found that BNP effect was attenuated similarly in TTC patients and acute HF patients. The residual effect was not associated with either patients' inflammatory status or catecholamine release.

In summary, this study identified that (1) In control subjects without diagnosed cardiovascular disease, BNP suppressed the release of the inflammatory modulator O_2^- from isolated neutrophils by attenuating NAD(P)H oxidase assembly; (2) This effect of BNP was lost in patients with acute HF, but recovers partially with chronic treatment of HF. (3) In TTC patients, attenuation of BNP effect was also present.

These data suggest that marked elevation of plasma concentration of BNP limits its physiological anti-inflammatory effects.

Declaration

I, Saifei Liu, certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Publications, presentations and awards related to the work conducted towards this thesis

Publications related to the work conducted in this thesis:

Liu, S, Ngo, DT, Stewart, S, Horowitz, JD & Chirkov, YY 2014 BNP suppression of neutrophil superoxide generation: mechanistic studies in normal subjects. Clin Exp Pharmacol Physiol. No. 41 Aug 12, pp 739-743.

Liu, S, Ngo, DT, Chong, CR, Amarasekera, AT, Procter, NE, Licari, G, Dautov, RF, Stewart, S, Chirkov, YY & Horowitz, JD 2015, 'Suppression of neutrophil superoxide generation by BNP is attenuated in acute heart failure: a case for 'BNP resistance'', Eur J Heart Fail, Feb 11. doi:10.1002/ejhf.242

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Liu, S, Ngo, DT, Chirkov, YY, Stewart, S & Horowitz, JD. Impaired B-type Natriuretic Peptide Effets on Neutrophil Burst in Heart Failure Patients. Presented at AHA scientific sessions 2013, Dallas, USA. *Circulation, vol. 128. A13335.*

Liu, S, Ngo, DT, Chong, CR, Chirkov, YY, Stewart, S & Horowitz, JD. Mechanisms of BNP interactions with neutrophil superoxide release: attenuation in acute and chronic heart failure. Presented at World Congress of Cardiology 2014, Melbourne, Australia. *Global Heart Journal 2014; 9: 1S: O066.*

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List of abbreviations

Abbreviation	Term
8-pCPT-cGMP	8-(4-Chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate
ACE	Angiotensin converting enzyme
ADMA	Asymmetric dimethylarginine
ADP	Adenosine diphosphate
AIx	Augmentation index
AngII	Angiotensin II
ANP	Atrial natriuretic peptide
ApoA-I	Apolipoprotein A-I
ASCEND-HF trial	Acute Study of Clinical Effectiveness of Nesiritide in Decompensated Heart Failure trial
AVP	Arginine vasopressin
BH4	Tetrahydrobiopterin
BNP	B-type natriuretic peptide
CAD	Coronary artery disease
CAT1-H	1-hydroxy-2,2,6,6-tetramethylpiperidin-4-yl-trimethylammonium chloride

cGMP	cGMP cyclic 3',5'- guanosine monophosphate
CGN	cGMP-gated ion channels
CGRP	Calcitonin gene-related peptide
CM-H	1- hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl pyrrolidine
CNP	C-tpye natriuretic peptide
CP-H	1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine hydrochloride
CRP	C-reactive protein
Cyto B	Cytochalasin B
db-cGMP	N2,2'-O-dibutyryl guanosine 3':5'-cyclic monophosphate
DMSO	Dimethyl sulfoxide
DPI	Diphenyleneiodonium
DPPIV	Dipeptidyl peptidase IV
EMPO	2-ethoxycarbonyl-2-methyl-3,4-dihydro-2H-pyrrole-1-oxide
eNOS	Endothelial nitric oxide synthase
EPR	Electron paramagnetic resonance
ESR	Electron spin resonance
ET-1	Endothelin-1

fMLP	*N*-formyl-methionyl-leucyl-phenylalanine
GM-CSF	Granulocyte/macrophage colony stimulating factor
GPx	Glutathione peroxidase
HBSS	Hanks' balanced salt solution
HCl	Hydrochloric acid
HDL	High-density lipoprotein
HF	Heart failure
H_2O_2	Hydrogen peroxide
HOCl/HClO	Hypochlorous acid
hs-CRP	High-sensitivity CRP
IBMX	3-Isobutyl-1-methylxanthine
IDE	Insulin-degrading enzyme
iNOS	Inducible nitric oxide synthase
IL	Interleukin
LAMs	Lipoarabinomannans
L-NMMA	N-monomethyl-L-arginine
LPS	Lipopolysaccharide
LTB4	Leukotriene B4

LV	Left ventricular
LVEF	Left ventricular ejection fraction
MCP	Monocyte chemoattractant peptide
MI	Myocardial infarction
mitoTEMPO-H	1-hydroxy-4-[2-(triphenylphosphonio)-acetamido]-2,2,6,6-tetramethylpiperidine
Mn-SOD	Manganese superoxide dismutase
MPO	Myeloperoxidase
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NEP	Neutral endopeptidase
NF	Nuclear factor
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO_2^-	Nitrite
NOS	Nitric oxide synthase
NPs	Natriuretic peptides
NPR-A	Natriuretic peptide receptor-A
NPR-B	Natriuretic peptide receptor-B

NPR-C	Natriuretic peptide receptor-C
NTG	Nitroglycerin
NT-proBNP	N-terminal pro B-type natriuretic peptide
NYHA class	New York heart association class
O_2^-	Superoxide
$OH^·$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
Ox-LDL	Oxidized low density lipoprotein
PAF	Platelet activating factor
PCWP	pulmonary capillary wedge pressure
pGC	Particulate guanylyl cyclase
PGD2	Prostaglandin D2
PGE2	Prostaglandin E2
PGF2α	Prostaglandin F2α
PGI2	Prostacyclin
PGs	Prostaglandins
PKC	Protein kinase C
PKGs	cGMP-dependent protein kinases

PMA	Phorbol myristate acetate
PP-H	1-hydroxy-4-phosphono-oxy-2,2,6,6-tetramethylpiperidine
Prx-3	Peroxiredoxin-3
RAAS	Renin-angiotensin-aldosterone system
ROS	Reactive oxygen species
SDMA	Symmetric dimethylarginine
Ser	Serine
sGC	Soluble guanylyl cyclase
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
TLR	Toll-like receptors
TM-H	1-hydroxy-4-methoxy-2,2,6,6-tetramethylpiperidine
TMT-H	*N*-(1-Hydroxy-2,2,6,6-tetramethylpiperidin-4-yl)-2-methylpropanamide
TNF-α	Tumor necrosis factor-α
TTC	Takotsubo cardiomyopathy
XO	Xanthine oxidase

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Chapter 1: Literature review

1.1 Introduction

B-type natriuretic peptide (BNP) belongs to the natriuretic peptide family, which has a wide range of biological effects, including natriuresis, diuresis, vasodilation, anti-fibrotic, anti-inflammatory, inhibition of renin-angiotensin-aldosterone system (RAAS) and inhibition of sympathetic nervous system activity (Kita et al. 1989; Lang et al. 1991; Wambach & Koch 1995). BNP is mainly involved in cardiovascular and renal homeostasis. The effects of BNP are mediated by the stimulation of particulate guanylyl cyclase (pGC), which leads to increased formation of cyclic 3',5'- guanosine monophosphate (cGMP) (Daniels & Maisel 2007; Murad 2006; Venugopal 2001; Wedel & Garbers 2001).

BNP is highly up-regulated in acute and chronic forms of congestive heart failure (HF), which remains a major healthcare problem (Anatoliotakis et al. 2013; Nieminen et al. 2006) and is associated with a marked increase in morbidity and mortality worldwide (Schrier & Abraham 1999). Systolic HF is usually defined on the basis that the heart muscle is unable to pump enough blood to meet the body's requirement for oxygen. However, this inadequacy is usually apparent only during exertion. Acute HF is a cardiac syndrome characterized by decreased cardiac output and acute increases in left ventricular (LV) end-diastolic pressure which results in increased ventricular stretch. In response to the increased ventricular wall stretch, BNP is released acutely.

Although early clinical trials have shown that systemic intravenous infusion of Nesiritide, a recombinant form of human BNP, improves acute decompensated HF haemodynamicly (Abraham et al. 1998; Chandra et al. 2008; Colucci et al. 2000; Hobbs, RE et al. 1996; Mills et al. 1999), the ASCEND-HF (Acute Study of Clinical Effectiveness of Nesiritide in

Decompensated Heart Failure) trial demonstrated that Nesiritide, relative to placebo, was not associated with a reduction in the rate of death and re-hospitalization after 30 days therapy in patients with acute decompensated HF. Moreover, Nesiritide therapy had no impact on dyspnea when utilized together with standard HF therapies (O'Connor et al. 2011).

In this chapter, the epidemiology and pathophysiology of congestive HF and the controversy regarding effects of BNP on congestive HF are reviewed.

1.2 Systolic HF

1.2.1 Terminology of HF

Systolic HF implies dysfunction of the heart in the sense that the heart is unable to pump enough blood and oxygen to support normal human activities (Figueroa & Peters 2006). From a clinical point of view, this includes the development of dyspnea, fluid overload, weakness and exercise intolerance.

1.2.2 Etiology of HF

Any structural or functional disorders of the heart including ischemic heart disease, valvular disease and disorders of heart muscle function can lead to systolic HF. Coronary artery disease (CAD) is the most common cause of HF. It has been claimed in many studies that the presence of CAD in patients with HF predicts a poor outcome (Smith et al. 2001). It is possible that this difference reflects the presence of extensive loss of functional myocardium through infarction.

1.2.3 Epidemiology of HF

With an increasing aging population worldwide, both the incidence and prevalence of HF is increasing. In the United States more than 75% of patients with HF are older than 65 years; while age-related changes in the cardiovascular system may predispose to the development of HF. Such changes include systolic hypertension and aortic stenosis.

In Australia, based on 2007-08 National Health Statistics self-reports, HF is estimated to affect about 277,800 Australians (1.4% of the population). More than half of the patients with the HF were females, with a prevalence of 1.7% (1.0% for males). The prevalence of HF is age dependent with an estimated prevalence of 2.6% in people aged 55–64 years to 8.2% in those aged 75 years and over (AIHW 2010).

1.2.4 Mortality and morbidity of HF

Evidence from a number of studies showed that the mortality rate is substantial in HF: 30-40% of HF patients die within a year and 60-70% die within 5 years of diagnose (Cowie et al. 2000; Ho, Anderson, et al. 1993; Ho, Pinsky, et al. 1993). Indeed the mortality rate for congestive HF is higher than most cancers (Stewart et al. 2001). In 2009, HF accounted for 17,900 deaths in Australia (AIHW 2012).

1.2.5 Pathophysiology of HF

Congestive HF is a cardiovascular syndrome characterized by complicated changes in cardio-renal, hemodynamic, and neurohormonal activations. Whether the injury to the myocardium resulted from CAD, hypertension, dilated cardiomyopathy, valvular disease or other causes, it leads to dysfunction of the left ventricle. Congestive HF is also accompanied by accentuated neurohormonal responses including both vasoconstrictor and

vasodilator hormone release (Table 1-1). Vasoconstrictor effect can also be induced by activation of the sympathetic nervous system, resulting in an increase in circulating catecholamines, which in turn potentially increased cardiac output; activation of the RAAS leads to retention of fluids and inflammatory activation; and increased circulating endothelin-1 (ET-1), a neurohormone that contributes to vasoconstriction, fibrosis and remodeling of the heart (Ruffolo & Feuerstein 1998). The vasodilators include natriuretic peptides (NPs), nitric oxide (NO), prostaglandins (PGs), kallidin and calcitonin gene-related peptide (CGRP) (Tziakas, Chalikias & Xatseras 2003). In congestive HF, the acute response of the neurohormone activation is compensatory. However, the long-term activation of these neurohormones leads to overexpression of biologically active molecules which in turn produce damage in the heart (hypertrophy, LV remodeling, apoptosis) and the circulation leading to worsening of cardiac function and progression of HF (Figure 1-1).

Table 1-1: Vasoconstrictor and vasodilator hormones that are activated in congestive HF.

Vasoconstrictor hormones	Vasodilator hormones
Norepinephrine	Natriuretic peptides
Epinephrine	Prostaglandins
Renin-angiotensin-aldosterone	Kallidin
Arginine vasopressin	Calcitonin gene-related peptide
Endothelin	Nitric oxide

Adapted from: (Tziakas, Chalikias & Xatseras 2003)

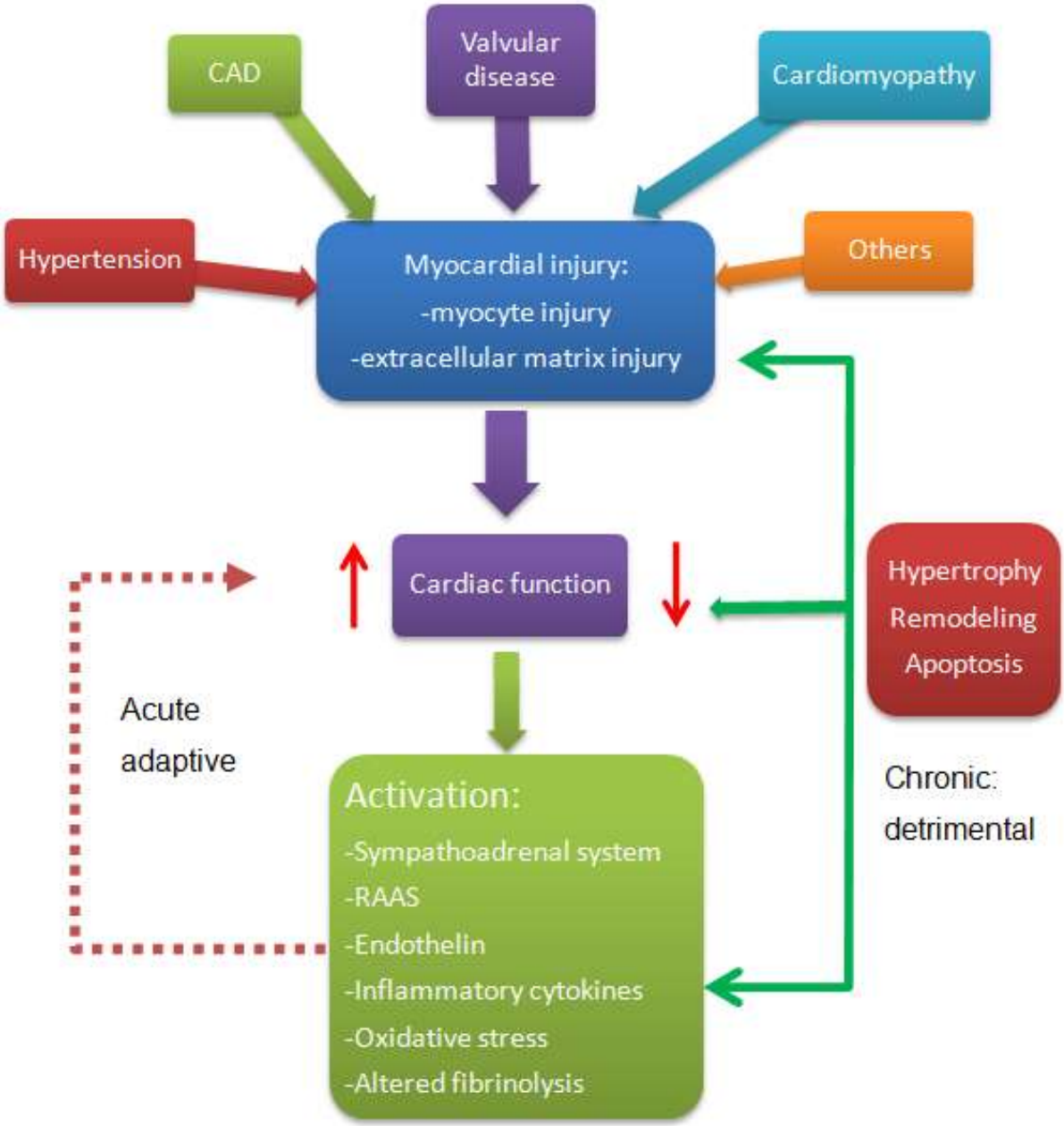

Figure 1-1: Pathogenesis of congestive HF.

Adapted from: (Braunwald 2013).

1.2.5.1 Neurohormonal responses in congestive HF

1.2.5.1.1 The vasoconstrictors

Although the clinical syndrome of congestive HF represents both systolic and diastolic dysfunction of the heart, the current understanding of the pathophysiology and the clinical evidence reflects a complex interaction between cardiac function and peripheral vascular function. For instance, alterations in the peripheral vasculature tone mediated by the effects of the neurohormonal vasoconstrictor systems can have substantial effects on overall cardiac performance in patients with congestive HF (Francis, GS et al. 1984).

The most important reported indicators of increased activity of the neurohormonal vasoconstrictor systems in congestive HF include increased plasma levels norepinephrine, epinephrine, ET-1, arginine vasopressin (AVP) and plasma renin activity, (Anker et al. 1997; Francis, GS et al. 1984; Nakamura, T et al. 2006).

The activity of the sympathetic nervous system is increased in HF (Chidsey, Harrison & Braunwald 1962; Cohn et al. 1984; Francis, GS et al. 1982; Levine et al. 1982). Augmented sympathetic activity in HF is an adaptive response initially but has harmful sequelae (Gaffney & Braunwald 1963). On one hand it increases cardiac output and redistributes blood flow. On the other hand renal vasoconstriction results in salt and water retention, which might be helpful by improve perfusion of vital organs. However, the chronically increased sympathetic activity down-regulates the β-adrenergic receptors in the failing heart leading to reduced responsiveness to inotropic and chronotropic stimuli (Bristow et al. 1982); sustained sympathetic stimulation, activates the RAAS and other neurohomones, which leads to progressive salt and water retention, vasoconstriction and oedema. ultimately increased pre- and after- load accelerates progression of HF (Yates, Beamish & Dhalla 1981).

The RAAS is directly involved in the homeostatic control of arterial pressure, tissue perfusion, fluid volume and vascular response to injury and inflammation (Atlas 2007).

The inappropriate activation of RAAS causes hypertension, sodium and water retention, inflammatory, thrombotic, and atherogenic effects that may lead to end-organ damage eventually (Brewster & Perazella 2004). The effects of the RAAS on target tissues are largely mediated by angiotensin II (AngII), which is generated in the presence of angiotensin converting enzyme (ACE), both in the circulation and in the tissues. In cardiovascular system, AngII effects are mediated by two receptors (AT1 and AT2 receptors) (Horiuchi, Akishita & Dzau 1999; Mehta, PK & Griendling 2007).

The RAAS is also critical to the regulation of nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase function. It has been reported that AngII increases the activity of NAD(P)H oxidase and production of superoxide (O_2^-) (Griendling et al. 1994). Furthermore, in AngII-dependent hypertension, the activity of components of NAD(P)H oxidase, especially NOX1, is increased. Activation of these pathways can be blocked by inhibiting renin and ACE or by blocking AngII receptors (Schramm et al. 2012). The pivotal role of ACE in the pathogenesis of cardiovascular diseases and the beneficial clinical effects of their inhibitors has been studied and demonstrated in many investigations, for review see (Ennezat et al. 2011).

AVP, also called antidiuretic homorne, is another vasoconstrictor and water-retaining hormone (Finley, Konstam & Udelson 2008). Several studies have shown a significant increase in plasma vasopressin levels in patients with HF and/or LV dysfunction (Francis, GS et al. 1990; Goldsmith et al. 1983; Lee et al. 2003; Szatalowicz et al. 1981). Within physiological levels, the vasoconstrictor effect of AVP is not associated with detectable pressor effects (Goldsmith 1987). Nevertheless, in patients with HF, exogenous AVP supplementation increases systemic vascular resistance and pulmonary capillary wedge

pressure, and decreases stroke volume and cardiac output dose-dependently (Goldsmith et al. 1986).

Endothelins, one of the potent vasoconstrictors that are released in HF, increase peripheral vasoconstriction by interacting with specific endothelin receptors, which are also found in the heart, kidney, adrenal gland and brain (Ferrara et al. 2002). Excessive endothelin release increases the after-load stress in HF and also has mitogenic effects, including myocyte hypertrophy and proliferation of the interstitial cardiac matrix. It has been reported that elevated ET-1 is an independent predictor of mortality in HF (Cleland, Dargie & Ford 1987).

1.2.5.1.2 Vasodilators

1.2.5.1.2.1 Natriuretic peptides

The NPs are a family of structurally similar but genetically distinct peptides. They have diverse effects in cardio-renal and endocrine homeostasis. The key observations that predicted the existence of NPs in biological system were reported almost sixty years ago when the heart was found to function as an endocrine organ. It was found that the atrial cells contained highly developed Golgi networks, which are similar to those observed in secretory cells (Kisch 1956); and later after that it was reported that atrial myocytes contain spherical, electron opaque granules (Jamieson & Palade 1964). Furthermore, Henry and colleagues (Henry, Gauer & Reeves 1956) conducted physiological experiments, which revealed that balloon distension of the atria associated with increased urination in dogs.

The first natriuretic peptide, discovered by de Bold and colleagues in 1981, was named atrial natriuretic peptide (ANP), which is secreted mainly from atrial myocytes (de Bold et

al. 1981). Shortly after this landmark discovery, a number of groups reported the purification and sequencing of atrial peptides of varying sizes that possessed natriuretic, diuretic, and/or smooth muscle relaxing activity (Currie et al. 1984; Flynn, de Bold & de Bold 1983; Kangawa, Fukuda, Kubota, et al. 1984; Kangawa, Fukuda, Minamino, et al. 1984; Kangawa & Matsuo 1984; Matsuo & Kangawa 1984; Misono et al. 1984). The second member of the family was purified and sequenced from porcine brain, and was therefore originally called brain natriuretic peptide (Sudoh et al. 1988). However, subsequent studies found that it is actually more concentrated in cardiac ventricles of patients with HF (Mukoyama et al. 1991; Mukoyama et al. 1990). Therefore, it is now often described as B-type natriuretic peptide (BNP). C-type natriuretic peptide (CNP), the third member of the NPs family, was purified in 1990s from porcine brain extracts based on its ability to relax smooth muscle (Kalra et al. 2001; Sudoh et al. 1990; Ueda et al. 1991). Soon after that, it was reported that CNP is distributed much wider in peripheral blood vessels (Heublein et al. 1992; Stingo et al. 1992) and exerts powerful vasorelaxation effects (Wei, Aarhus, et al. 1993).

All these three NPs contain a central loop with a conserved sequence CFGXXXDRIXXXXGLGC where X is different amino acids within each of the three peptides (Cowie & Mendez 2002), and this 17-amino-acid disulfide-linked ring is the major component for biological activity (Figure 1-2). ANP, BNP, and CNP are expressed in the tissues as prepro-hormones. The signal sequences are cleaved by different enzymes to form pro-ANP, pro-BNP, and pro-CNP, and then the peptides are processed proteolytically to form active peptides. Corin is the responsible enzyme for pro-ANP cleavage to form ANP (Yan et al. 2000). The enzymes responsible for BNP cleavage are furin and corin (Sawada et al. 1997; Yan et al. 2000). And also furin can cleavage the pro-

CNP to form a 53-amino-acid peptide (Wu, C et al. 2003). Another product of pro-CNP cleavage is a 22-amino-acid form and the enzyme responsible for this process has not been identified yet (Figure 1-2). The effects of NPs are mediated through natriuretic peptide receptors mainly on endothelial cells, vascular smooth muscle cells and other cells in large vessels, kidneys, adrenal glands and the brain. Interaction of NPs with their receptors plays a pivotal role in physiology and pathophysiology of hypertension and cardiovascular disorders.

Three receptors have been identified for NPs: natriuretic peptide receptor-A, B, C (NPR-A, NPR-B, and NPR-C). The alternative names of these receptors are pGCs (pGC-A, pGC-B) and the clearance receptor, or NPR1, NPR2, and NPR3, respectively. NPR-A catalyzes the synthesis of cGMP by binding of ANP or BNP (Figure 1-3). It contains an extracellular ligand-binding domain, a single membrane-spanning region, intracellular kinase homology domain, dimerization, and carboxyl-terminal guanylyl cyclase catalytic domains (Potter & Hunter 2001). NPR-B is homologous to NPR-A, but is activated by CNP. Most known physiological effects of NPs are mediated by these two receptors. The well-known physiological functions associated with the activation of NPR-A are diuresis, natriuresis, vasorelaxation, antagonism of the RAAS, and endothelial extravasation (Potter, Abbey-Hosch & Dickey 2006). All three NPs also bind NPR-C with similar affinities, and NPR-C is a disulfide-linked homodimer that is homologous to the extracellular domains of NPR-A and NPR-B, but consisted with only 37 intracellular amino acids. Overall, studies indicate that the primary role of NPR-C is to clear NPs from the extracellular environment via a receptor-mediated internalization and degradation process (Nussenzveig, Lewicki & Maack 1990). In addition to undergoing receptor-mediated degradation, NPs are also

metabolized by extracellular proteases, such as neutral endopeptidase, neprilysin (NEP) and insulin-degrading enzyme (IDE) (Potter 2011).

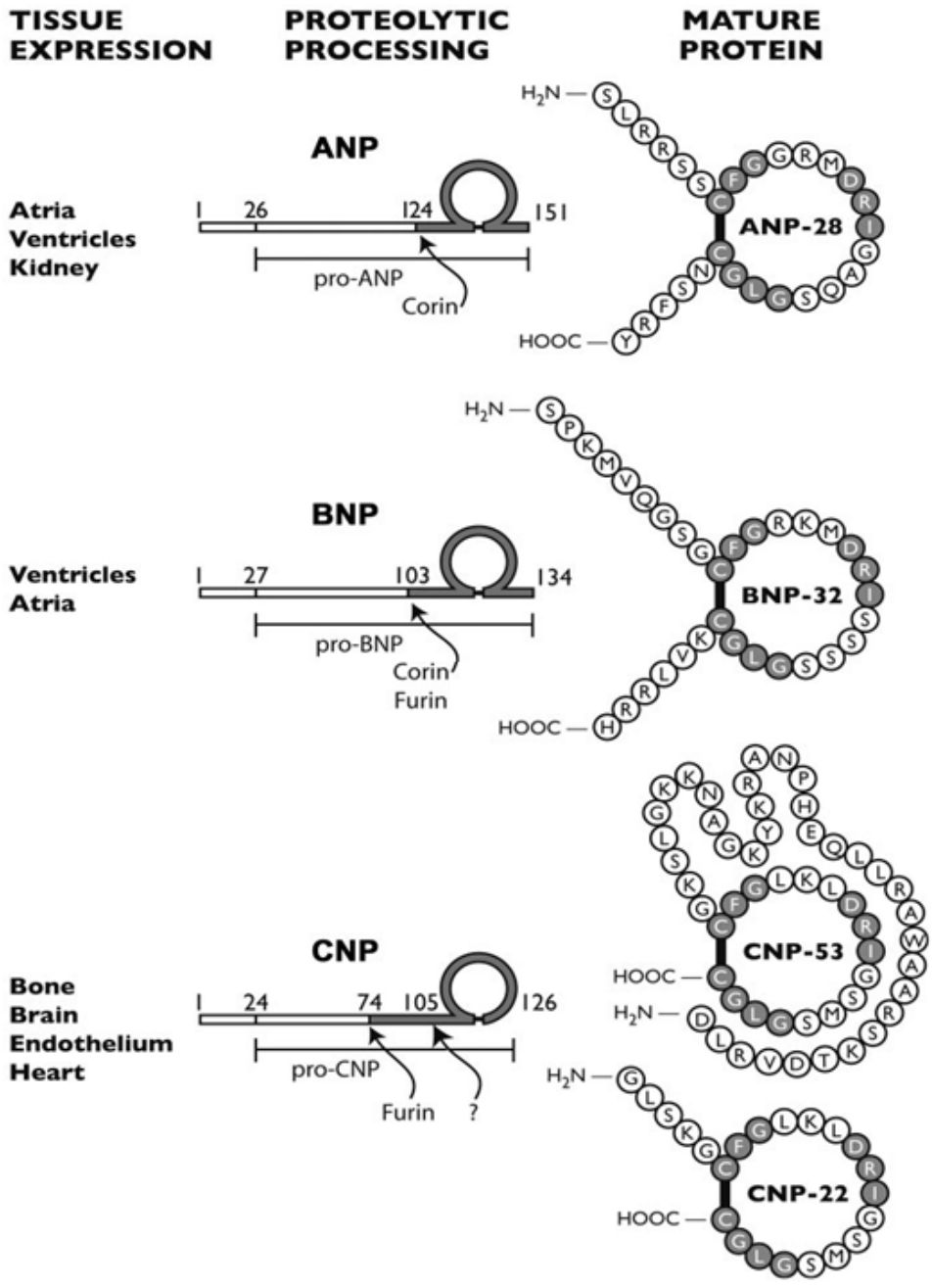

Figure 1-2: Natriuretic peptide expression, processing, and structure.

Adapted from: (Potter, Abbey-Hosch & Dickey 2006).

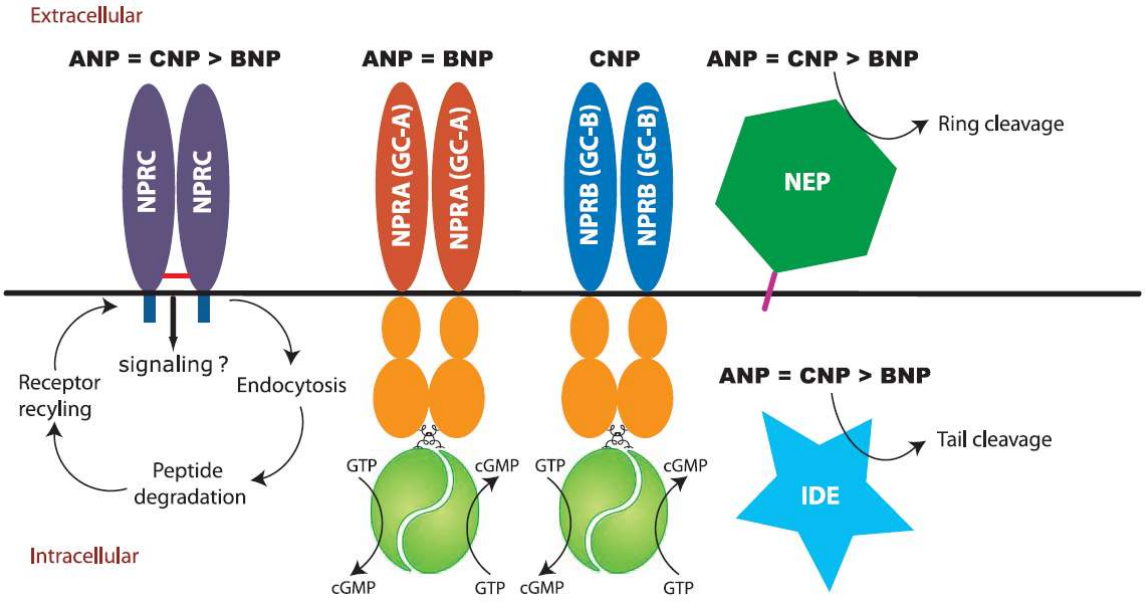

Figure 1-3: NPs bind multiple cell surface proteins.

Adapted from: (Potter 2011).

1.2.5.1.2.2 Nitric oxide

Nitric oxide (NO) is known to play an important role in regulation of cardiac function as well as other physiological effects. Although it is partially produced by reduction of nitrite anion (NO_2^-) (Hematian, Siegler & Karlin 2012), it is largely synthesized enzymatically by converting L-arginine to L-citruline and NO in the presence of NO synthase (NOS) (Moncada, Palmer & Higgs 1989; Palmer, Ashton & Moncada 1988; Palmer et al. 1988).

The enzymes responsible for the production of NO are the 'endothelial', 'neuronal' and 'inducible' isoforms of NOS (eNOS, nNOS and iNOS, respectively) (Balligand et al. 1995; Balligand et al. 1994; Simmons et al. 1994; Singh, K et al. 1994; Xu et al. 1999). Several other factors are also essential for the generation of NO from arginine by NOS, such as

tetrahydrobiopterin (BH4) and heme at the N-terminal catalytic oxygenase domain, together with flavins and NAD(P)H at the C-terminal reductase domain.

The major functions of NO include vascular smooth muscle relaxation and inhibition of platelet aggregation (Moncada, Radomski & Palmer 1988). To exert its beneficial effects on the cardiovascular system, NO acts primarily via the activation of soluble guanylate cyclase (sGC) which results in the production of cGMP and hence to vasodilatation, anti-inflammatory and anti-apoptotic effects as well as limitation of functional and structural remodeling of the myocardium (Cotton, Kearney & Shah 2002). It should be noted that NO may induce protein nitrosylation, potentially altering protein function and thus exerting sGC-independent effects. Also, if generated in excessive amounts, NO can be cytotoxic (Drapier, Wietzerbin & Hibbs 1988; Hibbs et al. 1988).

1.2.5.1.2.3 Prostaglandins

The vasodilator PGs: prostaglandin E2 (PGE2), prostacyclin (PGI2), prostaglandin D2 (PGD2), and prostaglandin F2α (PGF2α) are mainly synthesized in the heart (including cardiomyocytes, the interstitium and coronary arterials) (Dzau et al. 1984; Mehta, J & Mehta 1985; Satoh, Ohyama & Hayashi 1981) and kidney (renal arterioles, glomeruli and some parts of the renal tubules) (Folkert & Schlondorff 1979; Hassid, Konieczkowski & Dunn 1979). Interestingly, vasodilator prostanoid synthesis is stimulated by NO in a sGC-independent manner (Goodwin, Landino & Marnett 1999). Activation of RAAS and sympathetic nervous system can also lead to increased production of PGs (Dzau et al. 1984; Satoh, Ohyama & Hayashi 1981). PGs are known to affect coronary perfusion and myocardial function. Furthermore, by directly inhibiting the sodium transport in the distal tubules, PGs promote sodium excretion and protect glomerular function during conditions

such as in HF which are associated with renal vasoconstriction (Dzau 1988; Schrier & Abraham 1999).

1.2.5.1.2.4 Calcitonin gene-related peptide

Calcitonin gene-related peptide (CGRP) is released primarily from sensory nerves and up-regulated in HF, and is released partially in response to NO (Bellamy et al. 2006). Two forms of CGRP have been identified, (αCGRP is encoded by the calcitonin gene while βCGRP is formed by another gene) and performed as vasodilators (Brain, MacIntyre & Williams 1986). However, αCGRP is more abundant and its vasodilator effect has been studied in many different tissues including cardiovascular system (Brain & Williams 1985). CGRP is localized in the heart, blood vessels and the nervous system. Short-term infusion of CGRP in patients with congestive HF is associated with beneficial effects such as decreased system and pulmonary vascular resistance and increased cardiac output (Anand et al. 1991). Another study also suggested similar cardiovascular beneficial effects of CGRP in patients with congestive HF (Gennari et al. 1990). Furthermore, a recent study demonstrated that α-CGRP plays a significant role in protecting against the development of transverse aortic constriction-induced HF by decreasing inflammation, cell death, and fibrosis (Li, JP et al. 2013).

1.3 Congestive HF: focus on oxidative stress

Substantial clinical and experimental evidence suggests the involvement of oxidative stress in the pathophysiology of congestive HF (Belch et al. 1991; Giordano 2005; Hill & Singal 1996; Rochette et al. 2011; Sagols & Priymenko 2011). Oxidative stress describes a condition of imbalanced antioxidant defenses and increased production of reactive oxygen species (ROS). ROS are highly reactive, including free radicals such as O_2^-, hydroxyl

radical (OH•) and NO and non-radical oxygen derivatives such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$). ROS release plays a pivotal role in vascular injury and reflects the interplay of a number of different cytokines which are increasingly expressed after injury, shear stress and mechanical disruption (Nerem 1993; Nerem et al. 1998; Nerem et al. 1993).

Under physiological conditions, ROS generation plays an essential role in intracellular signaling pathways. However, when ROS are produced at markedly increased rates, this can cause cellular dysfunction, protein and lipid peroxidation, and DNA damage and then result in irreversible cell damage (Figure 1-4) (Seddon, Looi & Shah 2007). ROS release plays an important role in different physiological and pathological processes. For example, ROS can impair the contractile function of the heart, activate a number of hypertrophy signaling kinases and transcription factors, and mediate cardiomyocyte apoptosis (Chang & Wu 2006; De Vito et al. 2010). Furthermore, ROS can stimulate cardiac fibroblast proliferation and activate the matrix metalloproteinases, resulting in the extracellular matrix remodeling (Lijnen, van Pelt & Fagard 2011).

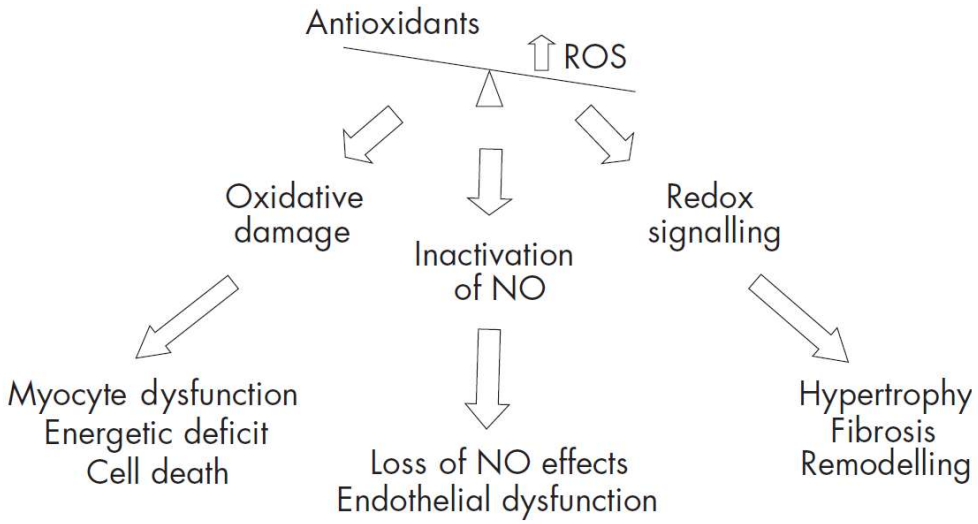

Figure 1-4: Main pathophysiological effects of oxidative stress in HF.

Adapted from: (Seddon, Looi & Shah 2007).

1.3.1 Redox stress inductor

Apart from the cardiomyocytes, which compose the majority of the cardiac cell mass, a variety of other cells also exist within the heart, including endothelial cells, smooth muscle cells, fibroblasts and neutrophils. All of them are potential sources of extensive ROS generation within the heart. Such ROS release may be derived from many sources (Figure 1-5), including the mitochondrial respiratory chain (Rojkind et al. 2002), cytochrome P450 monoxygenase (Rojkind et al. 2002), lipoxygenases, xanthine oxidase (XO) (Ekelund et al. 1999), uncoupled endothelial NOS (Xia, Y et al. 1998), and NAD(P)H oxidases (Seshiah et al. 2002). Some of these intracellular sources of ROS have been involved in the pathogenesis of HF either directly or indirectly.

Mitochondria represent the major energy supplier within the myocardium. However, mitochondria have also been implicated as a major source of extensive ROS generation. Mitochondrial respiration generates ROS via single electron transportation to oxygen molecules. Small amounts of ROS can be converted to H_2O_2 by manganese superoxide dismutase (Mn-SOD) which is a principal scavenger enzyme in the mitochondrial matrix. Thus, oxidative stress in this case can be due to increased ROS generation in the mitochondria, decreased dismutation of the oxygen free radical by endogenous mechanisms (Sam et al. 2005), or decreased detoxification of H_2O_2. It has been reported that mitochondrial dysfunction in the failing heart is associated with decreased enzyme activity of complex sites of the electron transportation chain (Ide et al. 1999), which results in increased O_2^- generation. Furthermore, heart specific Mn-SOD deletion results in

progressive HF due to significant degeneration of cardiac muscle (Li, YB et al. 1995). Thus, increased mitochondrial ROS production represents an important transition from physiological role towards a key role in pathogenesis of HF.

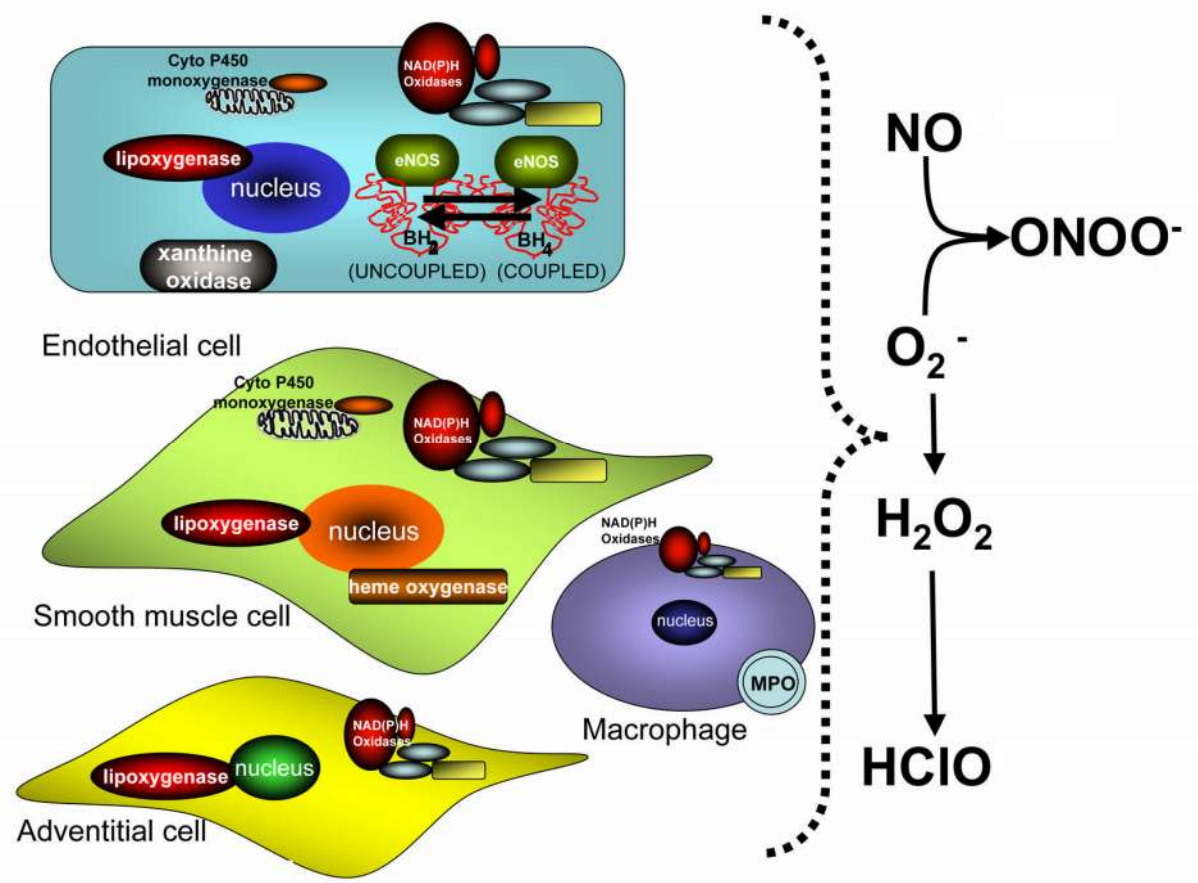

Figure 1-5: Sources of ROS in vascular cells.

NO nitric oxide, H_2O_2 hydrogen peroxide, O_2^- superoxide, HClO hypochlorous acid (HOCl), $ONOO^-$ peroxynitrite. Adapted from: (Papaharalambus & Griendling 2007).

Xanthine oxidase (XO), a molybdenum-containing enzyme that produces O_2^- in the catalysis of the terminal steps in purine metabolism, has been implicated as a pathogenic

factor in HF. XO is expressed in endothelial cells (Rouquette et al. 1998), but also circulating in the plasma in substantial concentrations (White et al. 1996) and myocardium (Abadeh, Case & Harrison 1993). XO catalyses the single electron reduction of molecular oxygen in the presence of xanthine, hypoxanthine and NADH, results in O_2^- formation. It has been shown that XO-mediated ROS production plays an important role in both experimental and clinical HF (Cappola et al. 2001; Ekelund et al. 1999). Under physiological conditions, activity of XO in myocardium is low, but it has been reported that both the expression and activity of XO is increased in canine rapid pacing-induced HF (Ekelund et al. 1999; Ukai et al. 2001) as well as in human end-stage HF (Cappola et al. 2001). Furthermore, application of the XO inhibitor, allopurinol, resulted in improvement of LV contractile function, which has been reported to be associated with a significant decrease in myocardial oxygen consumption and improvement in myocardial efficiency and contractility (Cappola et al. 2001; Ekelund et al. 1999; Ukai et al. 2001). On this basis, allopurinol has been recently developed as a prophylactic anti-anginal agent (Noman et al. 2010).

Uncoupled eNOS can lead to excess ROS production through the oxidation of the NOS essential cofactor BH4 (Landmesser et al. 2003). Physiologically, eNOS is tightly regulated and generates NO with the involvement of NAD(P)H, L-arginine, molecular oxygen, flavin adenine dinucleotide, and flavin mononucleotide. Under conditions of reduced BH4 or L-arginine, eNOS becomes structurally unstable and generates O_2^- (Landmesser et al. 2003; Vasquez-Vivar et al. 1998; Wever et al. 1997; Xia, Y et al. 1998). Increased oxidative stress can lead to BH4 oxidation (and hence inactivation) and can result in the uncoupling of eNOS (Landmesser et al. 2003). Furthermore, O_2^- release can result in $ONOO^-$ formation and resultant protein nitration.

ROS can be generated also via NAD(P)H oxidase in cadiovascular cells, as well as in activated leukocytes. The NAD(P)H oxidase contains a catalytic unit called NOX and the regulatory subunits p47phox (is heavily phosphorylated during enzyme activation), p67phox, p40phox, p22phox and the small GTPases Rac1and Rac2. NOX together with p22phox, form a heterodimeric cytochrome which is the site of electron transfer from NAD(P)H to molecular oxygen (O_2), resulting in the formation of O_2^-. Five different NOX isoforms have been identified (NOX1-5): each of them are encoded by different genes (Lambeth 2004).

In the cardiovascular system, the main isoforms present are NOX1, NOX2, NOX4, and NOX5 (Figure 1-6). NOX1 and NOX2 (also known as gp91phox in phagocytes) require the assembly of cytosolic regulatory subunits with the cytochrome to activate O_2^- production, while NOX4 activation does not require the cytosolic subunits. NOX1 is most abundantly expressed in vascular smooth muscle cells (Lassegue et al. 2001) and to a lesser extent in endothelial cells (Sorescu et al. 2004). NOX2 is largely expressed in cardiac myocytes, endothelial cells (Gorlach et al. 2000), aortic fibroblasts (Chamseddine & Miller 2003) and phagocytes. NOX4 is the most widely expressed isoform in endothelial cells, cardiac myocytes, and fibroblasts (Tsutsui, Kinugawa & Matsushima 2011). NOX2 and NOX4 are probably the most important isoforms in the context of the diseased myocardium. Recent studies have indicated that NOX4 plays an important role in mediating cardiac dysfunction via its role in increased ROS production and cardiac remodeling following pressure overload and aging (Ago, Kuroda, et al. 2010; Ago, Matsushima, et al. 2010; Kuroda et al. 2010). It has been shown that NAD(P)H oxidase activity is significantly increased by several stimuli that are important to the pathophysiology of HF, including mechanical stretch, Ang II, ET-1, and tumor necrosis

factor-α (TNF-α) (Griendling et al. 1997; Heymes et al. 2003). Doerries et al. (2007) demonstrated that p47phox-/- mice exhibited less LV remodeling and resultant LV dysfunction post myocardial infarction (MI).

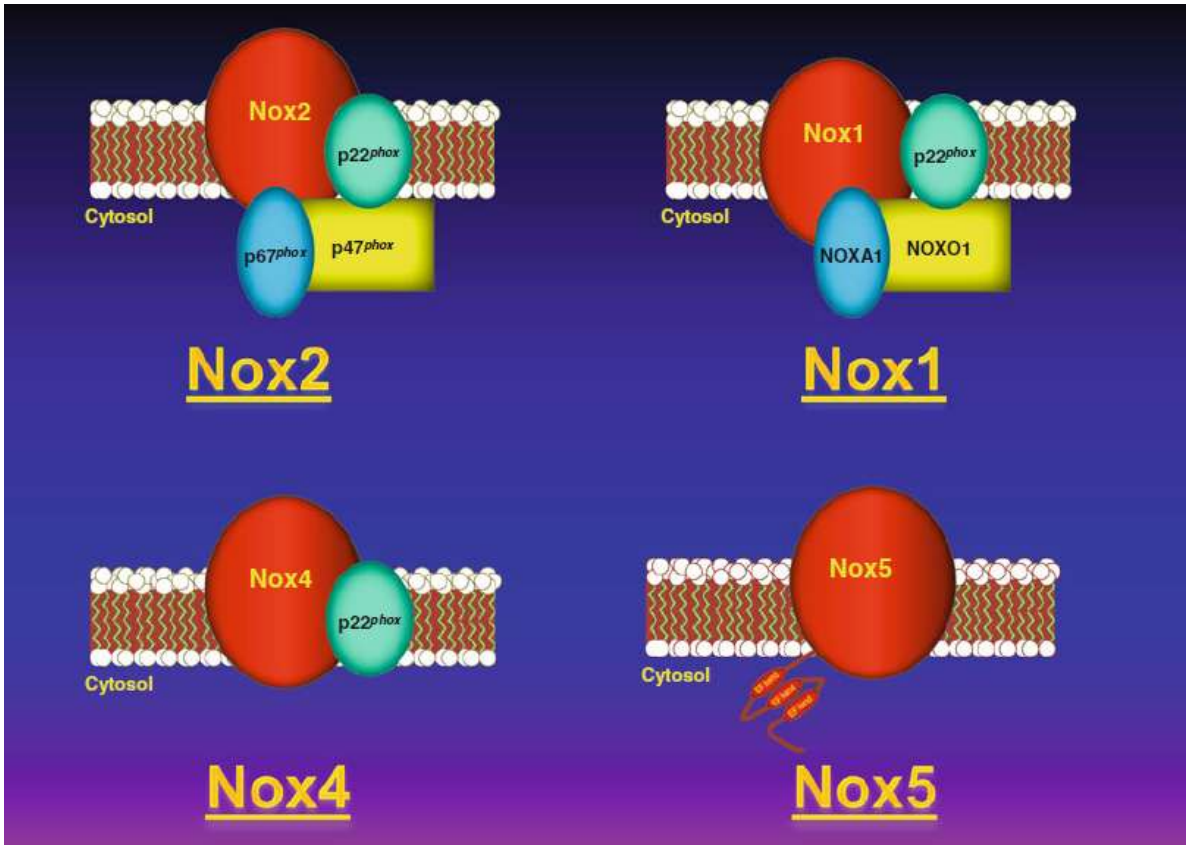

Figure 1-6: Representation of NAD(P)H oxidase family.

NOX1 and NOX2 require p22phox and the indicated cytosolic regulatory subunits for activation. NOX4 requires only p22phox. NOX5, on the other hand, is regulated by calcium binding through its N-terminal EF motifs. Adapted from: (Cifuentes-Pagano, Csanyi & Pagano 2012).

1.3.2 Focus on role of neutrophils

Neutrophil recruitment and activation is considered one of the defense mechanisms of innate immunity. In response to inflammatory mediators such as bacterial lipopolysaccharides (LPS), cytokines, and complement factors, neutrophils adhere to the vessel wall before migrating to the affected tissues. Neutrophil infiltration of the infarcted heart tissue after MI was described more than a century ago (Baumgarten 1898). A number of factors contribute to potential ischemic-reperfusion injury, including free radicals produced by infiltrating neutrophils, release of matrix-degrading enzymes and reduced activity of antioxidant enzymes. In addition, neutrophils are also involved in adverse cardiac remodeling and neointimal formation post MI. It has been reported that neutrophil depletion has beneficial effects during experimental infarction and reperfusion in animal models (Litt et al. 1989; Lucchesi 1990) and in clinical trials as well (Hayashi et al. 2000; Sawa et al. 1994). Furthermore, leukocytes have been suggested to be important in the pathogenesis of HF based on the finding that plasma levels of myeloperoxidase (MPO) were directly correlated with the severity of HF and were independent predictors of outcomes in these patients (Tang et al. 2007).

1.3.2.1 Neutrophil respiratory burst

The neutrophil respiratory burst, as well as neutrophil degranulation is fundamentally a defensive response to tissue damage in response to mechanical, chemical and infectious stimuli (Babior 1978; Klebanoff, S. J. 1980; Metschnikoff 1891). This process is tightly regulated in normal circumstances, resulting in leukocyte migration to the damaged area. Recruitment and activation of neutrophils will result in the production of large quantities of bactericidal molecules including ROS, which is beneficial for the tissue in the process of host defense. However, in the cardiovascular system the release of ROS can be

disadvantageous. The neutrophil respiratory burst can be triggered by a number of different inflammatory stimuli to produce O_2^-, which is important in redox signaling and plays an important role in development of pathophysiological conditions such as hypertension, ischemia-reperfusion injury, inflammation and atherosclerosis (Griendling, Sorescu & Ushio-Fukai 2000); O_2^- inactivates NO and counteracts its vasodilatory and anti-inflammatory effects. Also, the interaction between O_2^- and NO generates $ONOO^-$, which induces cellular injury.

The enzyme primarily responsible for the O_2^- formation in neutrophils is NAD(P)H oxidase (NOX2) (McPhail, Clayton & Snyderman 1984). Neutrophil NADPH oxidase can be activated by a number of factors including soluble proinflammatory cytokines, such as *N*-formyl peptides, C5a, platelet activating factor (PAF) and particulate stimuli. Priming of neutrophils occurs by adherence to biological surfaces, and circulating inflammatory mediators such as TNF-α and interleukin 6 (IL-6) largely increase their response in vitro. During the respiratory burst, the NAD(P)H oxidase acts as an electron donor to reduce oxygen to O_2^- (Babior 1999), which then dismutates to H_2O_2 and can be further processed to generate other ROS. Furthermore, the activation of the NAD(P)H oxidase in response to agonists leads to phosphorylation and subsequent translocation of several cytosolic NAD(P)H oxidase components to the membrane bound cytochrome (DeLeo & Quinn 1996), which are dependent on the production of phosphatidic acid by phospholipase D, p47phox phosphorylation (protein kinase C (PKC) dependent assembly of NAD(P)H oxidase), and guanine nucleotide exchange of Rac-GDP to form Rac-GTP (Babior, Lambeth & Nauseef 2002). The priming and activating agents of ROS production by neutrophils are summarized in Table 1-2.

Table 1-2: Priming and activating agents of ROS production by neutrophils.

	Agents	Priming	Activation
Cytokines	TNF-α	+++	+?
	GM-CSF	+++	-
	IL-1β	++	-
	IL-8	++	+?
	IL-15	+	-
	IL-18	++	-
TLR agonists	LPS	+++	-?
	LAMs	++	-
	Lipopeptide	++	-
	Flagellin	++	-
	R848	++	-
	Zymosan	++	+?
Chemoattractants	fMLP	++	++
	Complement C5a	+	++
	LTB4	++	+
	PAF	++	+
Chemical agents	PMA	+	+++
	A23187	++	+
	Cyto B	++	-
Others	Peroxynitrite	++	-
	Proteases	++	-
	Adhesion	++	-
	Fibronectin	+	-
	Substance P	++	-

A23187 calcium ionophores (ionomycin), Cyto B cytochalasin B, fMLP N-formyl-methionyl-leucyl-phenylalanine, GM-CSF granulocyte/macrophage colony stimulating factor, IL interleukin, LAMs lipoarabinomannans, LPS Lipopolysaccharide, LTB4

leukotriene B4, PAF platelet activating factor, PMA phorbol myristate acetate, R848 (a TLR7 and TLR8 agonist), TNF-α tumor necrosis factor α. − no effect, + weak effect, ++ moderate effect, +++ strong effect, ? contradictory data reported in the literature. Adapted from: (El-Benna, Dang & Gougerot-Pocidalo 2008).

Phosphorylation is an important process in the activation of the NAD(P)H oxidase. The phosphorylation of p47phox, p67phox and p40phox as well as a membrane bound component has been implicated in the activation process. More importantly, the activation of NAD(P)H oxidase is assisted by an extensive p47phox phosphorylation on several serine residues located in the polybasic region of the carboxy-terminal of the protein which is surrounded by serines 303 to 379 (el Benna, Faust & Babior 1994; El Benna et al. 1996). Among these different serines, serine379 (Ser379) phosphorylation is shown to be necessary for both the translocation of p47phox and the activation of the oxidase (Faust et al. 1995). However, it has been demonstrated that p47phox can be inactivated by mutation of a pair of serines to alanine, such as mutation of Ser303 and Ser304 which are known to be phosphorylated during oxidase activation, decreases oxidase activity dramatically (Inanami et al. 1998). Johnson et al. reported that the oxidase activity and phosphorylation of p47phox is greatly reduced in p47phox-deficient B lymphoblasts expressing the p47phox Ser359A/Ser370A or p47phox Ser359K/Ser370K double mutation compared with the same cells expressing wild type p47phox (Johnson et al. 1998). All of these, together with previous studies, suggest that oxidase activation requires the sequential phosphorylation of at least two serines on p47phox and the translocation of p47phox to the membrane (Babior 1999).

It has been shown that the pro-inflammatory cytokines, granulocyte/macrophage colony

stimulating factor (GM-CSF) and TNF-α are able to induce partial phosphorylation of p47phox on a major peptide, and enhance *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) induced p47phox phosphorylation of other sites (Dang et al. 1999; Dewas et al. 2003). Utilizing site-directed mutagenesis of Ser345 and the competitive inhibitory peptide containing the Ser345 sequence, Ser345 was identified in neutrophils as the phosphorylation site for TNF-α and GM-CSF (Dang et al. 2006), by phosphorylation of p47phox on Ser345 is thought to be a critical mechanism for the priming of ROS production by neutrophils. LPS and PAF has also been shown to induce partial phosphorylation of p47phox (Brown et al. 2004; DeLeo et al. 1998).

In addition to ROS, neutrophils may induce damage to the reperfused tissue through other mechanisms as well. The degranulation products of neutrophils such as proteases, collagenases, lipoxygenases, phospholipases and MPO can all contribute to neutrophil-mediated tissue damage.

1.3.2.2 Myloperoxidase

As mentioned above, MPO is another important enzyme released from neutrophils during the neutrophil respiratory burst (Klebanoff, S.J. 1991). MPO is a heme-containing enzyme that metabolises H_2O_2 to produce hypochlorous acid (HOCl) and other reactive oxidants which cause protein halogenation, nitration, and oxidative cross-linking (Figure 1-7). HOCl can chlorinate, nitrate or oxidize a variety of target molecules; reactions of HOCl with amines or ammonia leads to increased chloramines, which are also powerful oxidants. Overall, HOCl is widely considered to be a major factor modulating neutrophil cytotoxicity (Badwey & Karnovsky 1980; Weiss 1989).

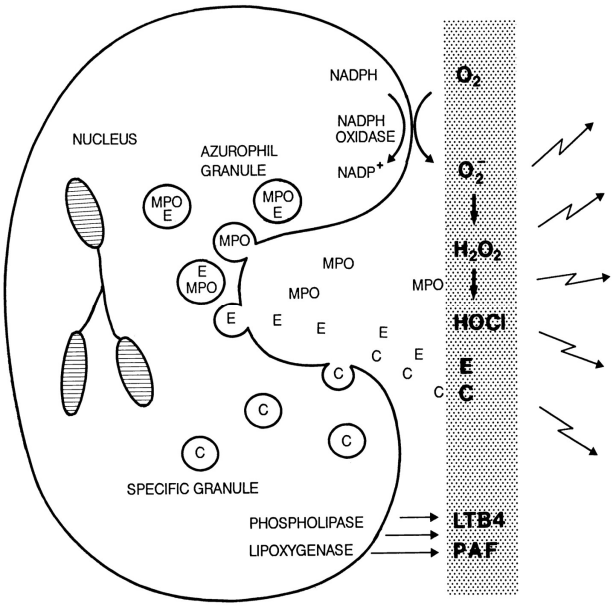

Figure 1-7: Schematic representation of inflammatory mediators with cardiotoxic potential released from activated neutrophils.

HOCl, hypochlorous acid; H_2O_2, hydrogen peroxide; MPO, myeloperoxidase; E, elastase; C, collagenase; LTB4, leukotriene B4; and PAF, platelet-activating factor. Adapted from: (Hansen 1995).

There is substantial evidence that MPO-catalyzed reactions contribute to the pathogenesis of many cardiovascular diseases, including initiation, propagation, and acute complication phases of the atherosclerotic process (DiDonato et al. 2014; Zheng et al. 2004). By reacting with HOCl or MPO, NO_2^- rapidly accelerates tyrosine nitration through formation of nitryl chloride and nitrogen dioxide radical (a reactive species that converts tyrosine to 3-nitrotyrosine) (Eiserich et al. 1996; van der Vliet et al. 1997). The NO-derived oxidation products generated by MPO, such as 3-nitrotyrosine and 3,5-dinitrotyrosine, are increased within human atheroma (Beckmann et al. 1994; Leeuwenburgh et al. 1997).

A recent study also showed that apolipoprotein A-I (apoA-I), the primary protein constituent of high-density lipoprotein (HDL), is a selective target for MPO-catalyzed nitration and chlorination *in vivo* (Zheng et al. 2004). Shao and colleagues (Shao, Pennathur & Heinecke 2012) demonstrated that nitration of apoA-I by MPO at tyrosine 192 transforms HDL into a more atherogenic molecule and therefore loss of its cardiovascular protective role. Thus increased plasma MPO concentrations represent a biomarker predicting adverse clinical outcomes of a number of cardiovascular diseases, including acute MI, reperfusion injury, stroke and HF (Loria et al. 2008; Tang et al. 2006).

1.3.3 Anti-oxidative mechanisms

Several antioxidant defense systems exist to balance the free radical formation in healthy subjects, including superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), thioredoxins, peroxiredoxins and also nonenzymatic antioxidants (e.g. glutathione).

SOD is believed to be one of the primary antioxidant defenses in vivo, catalyzing the dismutation of O_2^- to O_2 and H_2O_2. It has been reported that Mn-SOD activity is reduced in failing heart (Sam et al. 2005).

GPx, another key antioxidant, catalyzes the reduction of H_2O_2 and hydroperoxides (Figure 1-8). It has also been shown that GPx is largely present in the heart, notably in the cytosolic and mitochondrial compartments (Le et al. 1993). Furthermore, GPx has a higher affinity for H_2O_2 than catalase and prevents the formation of OH• and other toxic radicals (Figure 1-8). GPx is thought to be more effective compared with SOD and catalase within the heart. Overexpression of the GPx gene attenuates myocardial oxidative stress, remodeling and failure in mice (Matsushima, Kinugawa, et al. 2006; Shiomi et al. 2004).

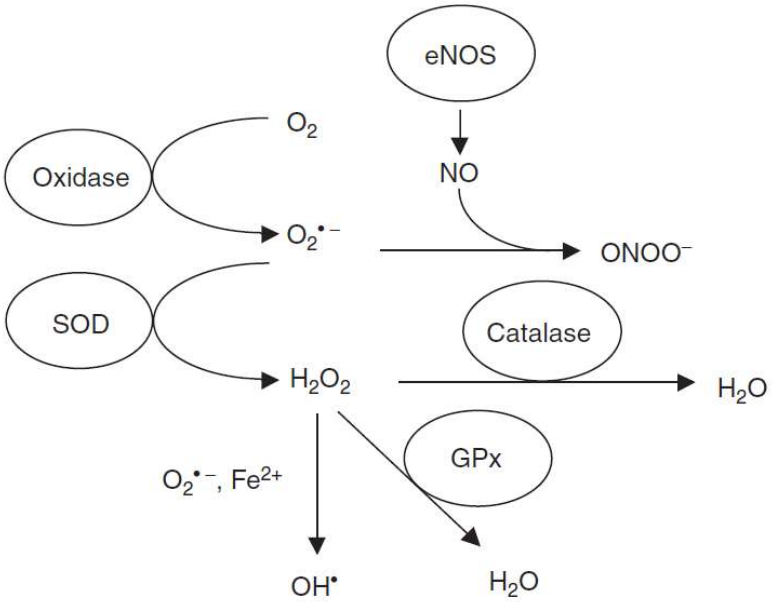

Figure 1-8: Reactions underlying the generation and degradation of reactive oxygen species.

Adapted from: (Paravicini, Drummond & Sobey 2004).

The thioredoxin system consisting of thioredoxin, thioredoxin interacting protein, thioredoxin reductase and NAD(P)H (Holmgren 1995), is one of the major cellular thiol-reducing and antioxidant systems that can protect cells by scavenging ROS. Oxidised thioredoxin is reduced by thioredoxin reductase and NAD(P)H. Two main thioredoxins have been described: - thioredoxin-1 and thioredoxin-2. It is reported that cytosolic thioredoxin-1 can protect the heart against oxidative stress and inhibit cardiac hypertrophy via its antioxidant activity (Yamamoto et al. 2003).

Peroxiredoxin-3 (Prx-3) is a member of peroxiredoxin family, which is an effective defense against oxidative stress. Prx-3 preferentially scavenges H_2O_2 together with thiol and $ONOO^-$ (Bryk, Griffin & Nathan 2000). Prx-3 is localized mainly in the mitochondria

(Kang et al. 1998) and studies involving in vivo transfer of the Prx-3 gene showed a protective role of Prx-3 on neurons against cell death induced by ROS release (Hattori et al. 2003). Furthermore, it has been recently demonstrated that overexpression of Prx-3 had beneficial effects in limiting post-MI remodeling and failure in mice. This effect is consistent with attenuation of oxidative stress, mitochondria DNA decline, and dysfunction (Matsushima, Ide, et al. 2006).

Regarding the leukocyte NAD(P)H oxidase, sulfhydryl groups play important roles. Previous studies have shown that naturally existing sulfhydryl blockers such as the aldehyde 4-hydroxynonenal inhibited NAD(P)H oxidase (Siems et al. 1997). Aldehyde is a major product of lipid peroxidation, therefore, inhibition of NAD(P)H oxidase by 4-hydroxynonenal is of physiological significance. NOX2-targeted inhibitory peptides such as NOX2 docking sequence-tat, which is a 18-amino acid peptide, have been shown to inhibit NAD(P)H oxidase activity in vivo and in vitro (Jacobson et al. 2003; Liu, J et al. 2003; Rey et al. 2001). Other peptide inhibitors targeting the other subunits of NAD(P)H oxidase have also been reported to act as effective antagonists, as well as small molecule inhibitors including diphenyleneiodonium (DPI), VAS2870 and VAS3947, fulvene-5, Gkt136901, ML171, celastrol (for review see (Cifuentes-Pagano, Csanyi & Pagano 2012)).

NO also exerts direct antioxidant effects by inhibiting the NAD(P)H oxidase through limiting the assembly of the oxidase during activation (Fujii et al. 1997). Nitrosothiols prevent the translocation of the cytosolic subunits p47phox and p67phox to the membrane, and therefore also inhibit NAD(P)H oxidase activation (Ding et al. 1996).

1.4 Congestive HF: focus on inflammatory activation

Inflammation has been implicated in the pathophysiology of HF in both animal models

(Sun, Y et al. 2002) and humans (Torre-Amione, Kapadia, Benedict, et al. 1996; Torre-Amione, Kapadia, Lee, et al. 1996). A number of signaling molecules are involved in mediating inflammatory responses, including PGs, C-reactive protein (CRP), soluble CD40 ligand, adiponectin, and inflammatory cytokines, such as TNF-a. Several studies have demonstrated increased expression and release in HF patients of inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-18, cardiotrophin-1 and Fas ligand, as well as chemokines including monocyte chemoattractant peptide (MCP)-1, IL-8 and macrophage inflammatory protein-1α (Adamopoulos, Parissis & Kremastinos 2001; Aukrust et al. 1999; Aukrust et al. 1998; Damas, Gullestad, et al. 2000; Torre-Amione, Kapadia, Benedict, et al. 1996; Torre-Amione, Kapadia, Lee, et al. 1996). Increased plasma levels of inflammatory cytokines and chemokines have been significantly correlated with worsening of cardiac function (Adamopoulos, Parissis & Kremastinos 2001; Aukrust et al. 1999; Aukrust et al. 1998; Damas, Eiken, et al. 2000; Testa et al. 1996; Torre-Amione, Kapadia, Benedict, et al. 1996; Torre-Amione, Kapadia, Lee, et al. 1996). The relationships between inflammatory mediators and symptoms of inflammation are summarized in Table 1-3. Moreover, these inflammatory mediators are also valuable prognostic markers in patients with chronic HF (Deswal et al. 2001; Torre-Amione, Kapadia, Benedict, et al. 1996; Ueland et al. 2005).

1.4.1 Cellular mechanisms

The increased circulating inflammatory cytokines in HF have multiple sources including several tissues and cell types. The myocardium itself may be an important source of the increased inflammatory mediators found in the circulation in HF. It has been demonstrated that the failing myocardium expresses increased levels of a range of inflammatory mediators, such as adhesion molecules, TNF-α, IL-6-related cytokines and chemokines (Damas, Eiken, et al. 2000; Eiken et al. 2001; Torre-Amione, Kapadia, Lee, et al. 1996).

Valen et al. claimed that the activation of transcriptional factor nuclear factor (NF)-κB, a major mediator of inflammation within the failing myocardium, has anti-apoptotic rather than pro-apoptotic effects (Valen, Yan & Hansson 2001). Thus, the release of these cytokines from the failing heart may not only contribute to the pathogenesis of HF, and other cells within the failing heart such as endothelial cells and fibroblasts may relate to the myocardial inflammation in HF.

Apart from the myocardium itself, several tissues and cells contribute to the inflammation condition in HF, including leukocytes, platelets, tissue macrophages and endothelial cells (Yndestad et al. 2006). Patients with chronic HF are characterized by increased expression of inflammatory mediators in circulating leukocytes. A number of studies have shown that mononuclear cells including T cells, B cells, NK cells and monocytes from the peripheral blood of HF patients have increased gene expression as well as release of inflammatory cytokines such as chemokines and ligands in the TNF superfamily (Conraads et al. 2005; Damas et al. 2001; Yndestad et al. 2002; Yndestad et al. 2003; Zhao & Xu 1999). Activated platelets can also release inflammatory mediators such as chemokines and soluble CD40 ligand (Aukrust et al. 1998; Damas, Eiken, et al. 2000; Ueland et al. 2005). It has been demonstrated that the platelet-derived mediators may induce inflammatory responses in leukocytes and endothelial cells nearby, which in turn will further stimulate platelet activation (Weber 2005). Endothelial cells are another source of inflammatory mediators, which is activated in chronic HF and results in enhanced expression of chemokines such as IL-8 and MCP-1, adhesion molecules and cyclooxygenase-2 as well as promoting leukocyte-endothelial interaction (Colombo et al. 2005; Tousoulis, Charakida & Stefanadis 2005). Furthermore, other sources of inflammatory cytokines have also been reported: clinical and experimental studies have shown that expression of cytokines (MCP-

1 and IL-6) is increased in the lung of HF patients (Mabuchi et al. 2002; Tonnessen et al. 2003), and that the pulmonary endothelium is another source of inflammatory mediators (Gaertner et al. 2003); moreover, elevated serum levels of TNF-α represent in part release from the liver in pacing-induced HF rabbits (Aker et al. 2003).

Table 1-3: Relationships between inflammatory mediators and symptoms of inflammation in cardiovascular diseases.

Symptom	Mediators
Vascular permeability	Vasoactive amines
	Bradykinin
	Leukotrienes C4,D4,E4
	PAF
	Complement (C3a and C5a)
	Substance P
	NO
Vasodilatation	NO
	PGI2, PGE1, PGE2, PGD2
	H_2O_2
Vasoconstriction	Tromboxane A2
	Leukotrienes C4,D4,E4
	O_2^-
Chemotaxis and leukocyte adhesion	Chemokines
	LTB4, HETE, lipoxins
	C5a
	Bacterial antigens
	O_2^-
Tissue and Endothelial damage	ROS
	iNOS
	Lyzosomal enzymes

PAF platelet activating factor, LTB4 leukotriene B4, NO nitric oxide, PG prostaglandin, O_2^- superoxide, H_2O_2 hydrogen peroxide, ROS reactive oxygen species, iNOS inducible nitric oxide synthase. Adapted from: (Guzik, Korbut & Adamek-Guzik 2003).

1.4.2 Humoral mechanisms

ROS release has recently been indicated to play a key role in modulating the release of other inflammation mediators, due to the expression of NAD(P)H oxidases in various tissues (Guzik et al. 2000). ROS produced by NAD(P)H oxidase can increase chemokine and cytokine expression (Kimura et al. 2003). Hypoxia and ischemia have also been found to induce inflammatory cytokines such as TNF-α, MCP-1 and IL-8 which probably through production of ROS with secondary activation of the transcription factor NF-κB in the endothelium, leukocyte subpopulations and ischemic and reperfused canine myocardium (Li, N & Karin 1999). Therefore, intracellular ROS may possibly act as second messengers in inflammatory signal transduction. Moreover, mechanical overload and shear stress may induce MCP-1, IL-1β and IL-8 expression in cardiomyocytes and endothelial cells (Okada et al. 1998; Shioi et al. 1997). Oxidized low density lipoprotein (Ox-LDL) has been reported to increase cytokine expression in monocyte-derived macrophages (Janabi et al. 2000); this process may play an important role in myocardial failure secondary to CAD. Furthermore, several other stimuli have been demonstrated to be involved, such as toll-like receptors (TLR), autoantibodies, heat-shock proteins, microbial antigens, endotoxins, hemodynamic overload and neurohormones etc. (Figure 1-9).

A variety of inflammatory cytokines are up-regulated in chronic HF. Inflammatory cytokines together with related mediators could be used as markers for risk stratification and prognostication in patients with HF. CRP has been used widely as an inflammatory biomarker in cardiovascular disease, based on its ability to reflect upstream inflammatory activity. Several studies have shown elevated CRP levels in association with HF development and adverse outcome. Recently, a study demonstrated that in patients with HF, enhanced CRP was associated with features of more severe HF and was independently associated with adverse outcome (Anand et al. 2005). Moreover, a substudy of the Controlled Rosuvastatin Multinational Trial in HF (CORONA) demonstrated a significant interaction between CRP and the effect of rosuvastatin on adverse events (McMurray et al. 2009). Furthermore, several studies suggest that TNF-α, IL-6 or IL-β can predict adverse outcome in these patients (Deswal et al. 2001; Orus et al. 2000). Therefore, assessing these cytokines in a larger population of HF patients may provide important pathophysiological insights.

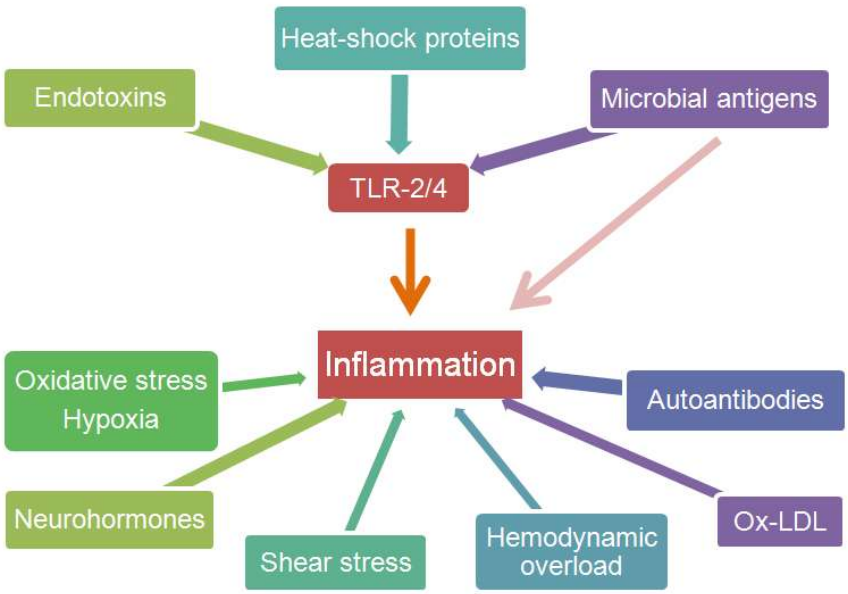

Figure 1-9: schematic representative of systemic inflammation in chronic HF.

Ox-LDL Oxidized low density lipoprotein, TLR toll-like receptors. Adapted from: (Yndestad et al. 2006).

1.5 CHF: focus on impairment of endothelial function

The vascular endothelium is a monolayer of cells between the circulating blood in the vessel lumen and the vascular smooth muscle cells. It regulates the peripheral blood flow and vasomotor tone through the production and release of a number of vasoactive factors and chemical signals (Petty & Pearson 1989; Vane, Anggard & Botting 1990). Generally speaking, the healthy endothelium maintains a non-adhesive luminal surface and has vasoconstrictor and vasodilator, growth inhibition, anticoagulation, anti-inflammatory, and antioxidant properties (Rubanyi 1993).

Vascular endothelial function plays a key role in the pathophysiology and the prognosis of HF (Katz et al. 2005). Endothelial dysfunction is an early and important characteristic of many vascular diseases. Considerable evidence has shown that patients with HF have abnormal endothelial function (Bonetti, Lerman & Lerman 2003; Fischer et al. 2005; Heitzer et al. 2005). The key factors modulating endothelial dysfunction are reduced bioavailability of NO (which can be caused by decreased eNOS expression of the endothelial cells), decreased substrate for NO formation, increased degradation of NO by ROS as well as excessive formation of ROS within the vascular wall (Pou et al. 1992; Shimokawa, Flavahan & Vanhoutte 1991; Wilcox et al. 1997).

In a rat model of HF, decreased eNOS expression and NO synthesis in the endothelium have been reported (Comini et al. 1996). In patients with HF, a reduction in nitrate excretion by the kidneys at rest and during exercise after arginine infusion, which reflects

impairment of NO generation, has also been reported (Katz et al. 1999). Moreover, NO resistance at the level of platelet aggregation has been described in HF (Anderson et al. 2004). Impaired endothelium-dependent vasodilation in patients with HF is thought to be associated with reduced activity of the L-arginine-NO synthetic pathway, increased degradation of NO by ROS, and hyporesponsiveness in vascular smooth muscle as well as decreased antioxidant defenses (Bauersachs et al. 1999; Katz et al. 1999).

Various components of endothelial dysfunction may be evaluated physiologically or biochemically. Asymmetric dimethylarginine (ADMA) is generated via protein catabolism and cleared by dimethylarginine dimethylaminohydrolase, which is a redox-dependent enzyme. ADMA can be assayed in either plasma or tissue samples (Murray-Rust et al. 2001). Increased plasma concentrations of ADMA have been found in HF patients, and this elevation is associated with poor cardiovascular outcomes (Duckelmann et al. 2007). Tissue sGC activity (Sakurada et al. 2008) is another potential modulator of endothelial function. Responses to acetylcholine (Halcox et al. 2002; Schachinger, Britten & Zeiher 2000) or salbutamol (Rambaran et al. 2008) are normally used as measures of "endothelial function" *in vivo*, while responses to nitroglycerin (NTG) or sodium nitroprusside (SNP), the NOS-independent sources of NO, are used to evaluate NO-based signaling pathways (Kasprzak, Klosinska & Drozdz 2006).

1.6 Focus on BNP in HF

1.6.1 Synthesis and storage of BNP

Although BNP was initially purified from porcine brain extracts (Sudoh et al. 1988) and given the name "brain natriuretic peptide", the highest concentration of BNP is present in the heart (Ogawa, Y et al. 1990). As a novel cardiac hormone, BNP is mainly synthesized

in and secreted from the ventricle (Mukoyama et al. 1991; Nakao et al. 1991; Ogawa, Y et al. 1991). The BNP gene is located on the distal short arm of chromosome 1, and encodes the prohormone proBNP, which is close to and upstream from the ANP gene (Tamura et al. 1996; Vila et al. 2008). In the secretory granules of atrial and ventricular myocytes, the BNP precursor protein (proBNP) coexists with ANP (Nakamura, S et al. 1991; Wei, Heublein, et al. 1993).

1.6.2 Release of BNP: physiology and pathology

Mean BNP concentration in venous blood are detectable at picomolar concentrations in normal subjects. Although BNP has a short half-life of only about 20 minutes in healthy subjects (Holmes et al. 1993; Mukoyama et al. 1991), plasma BNP concentrations do not generally show rapid fluctuation in healthy subjects, unlike ANP. ANP is released immediately from atrial storage granules in response to atrial wall stretching, whereas BNP secretion is largely controlled at the transcriptional level (de Bold, Bruneau & Kuroski de Bold 1996; Magga et al. 1997). A longer term stimulus is generally associated with increased plasma BNP concentrations by increasing the rate of synthesis as well as secretion (Lang et al. 1991). Nevertheless, some stimuli can increase plasma BNP concentrations within minutes. Intense exercise produces a moderate increase in plasma BNP concentration, and a greater increase was observed in individuals with LV hypertrophy or HF (Friedl et al. 1999; Wijbenga et al. 1999; Yamazaki et al. 2000).

Although stretch and wall tension are likely to be important in controlling production and secretion of BNP, the precise mechanisms are still unclear. BNP up-regulation is seen in several pathological states, such as hypertrophic cardiomyopathy, dilated cardiomyopathy (Hasegawa et al. 1993), and other forms of HF (Wei, Heublein, et al. 1993). Plasma BNP concentration is also elevated in pulmonary hypertension, which is thought to be due to

secretion from the right ventricle (Cowie & Mendez 2002). This plasma BNP elevation is well correlated with right ventricular end-diastolic pressure and right ventricular muscle mass (Nagaya et al. 1998). Reduced oxygen tension has also been reported to stimulate BNP gene expression in cultured ventricular myocytes (Casals et al. 2009; Goetze et al. 2003; Xia, WJ et al. 2011).

Although the main source of circulating BNP is the heart, plasma levels of BNP can be affected by extra cardiac disease states and also by factors that affect clearance of the peptide, such as variation in neutral endopeptidase and vasopeptidase inhibitors. The effect of various disease conditions on the plasma concentration of NPs is summarized in (Table 1-4).

1.6.3 Physiological actions of BNP

BNP acts through binding to NPR-A which results in the generation of the second messenger cGMP (Garbers 1992). The intracellular cGMP is believed to produce cellular and physiological responses by interacting with cGMP-dependent protein kinases (PKGs), cGMP-gated ion channels (CGN), and cGMP-regulated cyclic nucleotide phosphodiesterases (Figure 1-10) (Lincoln & Cornwell 1993), which means that cGMP regulates a number of intracellular processes, such as vascular smooth muscle relaxation (Rivero-Vilches et al. 2003), protection from oxidant damage (Grosser & Schroder 2003), proliferation (Morbidelli et al. 1996), Ca^{2+} handling by the sarcoplasmic/endoplasmic reticulum ATPase (Lau et al. 2003), and the control of endothelial permeability (Kiemer et al. 2002; Pedram, Razandi & Levin 2002).

Table 1-4: The effect of various disease states (other than HF) on the plasma concentrations of the cardiac NPs:

Disease		Likely effect on plasma concentrations
Pulmonary disease	Chronic pulmonary disease	Increased in cor pulmonale or pulmonary disease with evidence of right heart "strain", especially in the presence of acute exacerbation of airways disease or severe hypoxia
Vascular disease	Systemic hypertension	May be increased (especially in presence of concentric LV hypertrophy with a typically 3-fold elevation of BNP)
	Pulmonary hypertension	Increased
Structural cardiac disease	Aortic or mitral stenosis	Increased
	Hypertrophic cardiomyopathy	Increased
Endocrine & metabolic disease	Thyroid disease	Increased in hyperthyroidism
	Cushing's syndrome (or exogenous glucocorticoids)	Increased
	Primary aldosteronism	Increased
	Addison's disease	May be increased in treated cases
	Diabetes mellitus	Possibly increased in patients with microalbuminuria or autonomic dysfunction
Hepatic cirrhosis with ascites		Increased
Renal failure (acute or chronic)		Greatly increased (decreases with hemodialysis)
Paraneoplastic syndrome		May be increased
Subarachnoid hemorrhage		Increased

(Adapted from: (Cowie & Mendez 2002).

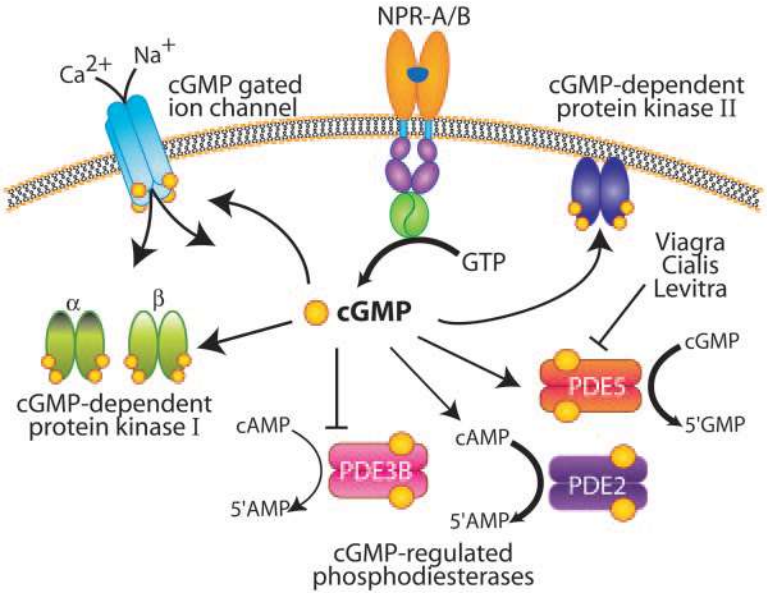

Figure 1-10: Cyclic GMP signaling pathway.

(Adapted from: (Potter, Abbey-Hosch & Dickey 2006).

On the other hand, BNP may function in parallel/competition with NO, which also releases cGMP through activation of sGC (Garg & Hassid 1989), and thus maintain cardiovascular and renal homeostasis (Moncada, Palmer & Higgs 1991). NO is produced by NOS, which has been identified in three isoforms, eNOS, nNOS and iNOS that are expressed in many tissues, including endothelium, vascular smooth muscle, specific segments of the nephron and the heart (Alderton, Cooper & Knowles 2001; Forstermann et al. 1991). In 1987, it was reported that ANP and SNP independently increase cGMP in cultured rat lung fibroblasts by activating pGC-A and sGC (Leitman et al. 1987). However, in recent studies, increasing evidence has accumulated of cross-talk between these two enzymes (Kotlo, Rasenick & Danziger 2010). ANP has also been reported to stimulate the NO signaling pathway via activation of NOS in primary cultures of human proximal tubular cells (McLay et al. 1995), rat kidney (Elesgaray et al. 2008), and rabbit ventricular myocytes

(William et al. 2008) via NPR-C. Furthermore, by comparing the vasorelaxing effects of ANP and CNP in aorta and mesenteric small arteries from wild-type and endothelial NOS knockout mice, in the presence of NPR-A antagonist, Madhani et al. proved that both NPR-A and NPR-B-linked GC pathways are modulated by NO-cGMP signaling (Madhani et al. 2003).

In HF, BNP has numerous potentially beneficial effects, including diuretic, natriuretic, vasodilating (Dries 2011; Okumura et al. 1995), decreasing renin-angiotensin system activation (Dries 2011; Struthers 1994), anti-fibrotic, anti-hypertrophic effects (Dries 2011) and inhibition of the synthesis of ET-1 (Stoupakis & Klapholz 2003). In patients with chronic HF, BNP improves central hemodynamics, including the cardiac index, through suppression of myocyte proliferation, cardiac growth, and compensatory hypertrophy of the heart (Stoupakis & Klapholz 2003).

1.6.4 Clearance of BNP

All three NPs are degraded through two well-characterized processes: (1) NPR-C-mediated internalization followed by lysosomal degradation; and (2) enzymatic degradation. It has been reported that the active BNP1-32 can be degraded by dipeptidyl peptidase IV (DPPIV), NEP, meprin and IED to form BNP3-32, BNP5-32, BNP8-32 and smaller degradation peptides (Figure 1-11) (Boerrigter et al. 2009; Boerrigter et al. 2007; Brandt et al. 2006; Muller et al. 1992; Pankow et al. 2007; Ralat et al. 2011; Toll et al. 1991). Studies in sheep have demonstrated that the enzymatic and receptor-mediated processes contribute equally to the degradation of ANP and BNP (Charles et al. 1996).

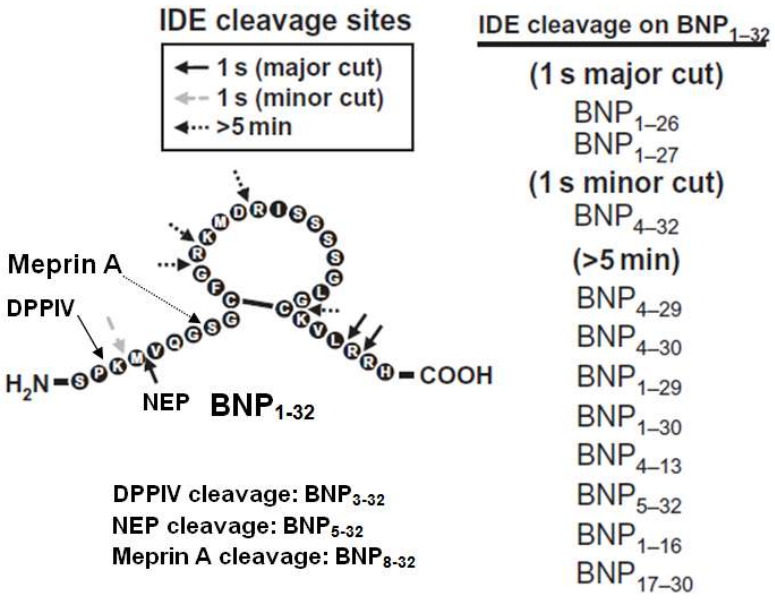

Figure 1-11: BNP1–32 degradation and cleavage sites.

DPPIV dipeptidyl peptidase IV, *IDE insulin-degrading enzyme, NEP neutral endopeptidase. Adapted from: (Ichiki, Huntley & Burnett 2013).*

1.6.5 Circulating BNP fragments

While it is known that BNP represents an enzymatic cleavage product of the 108 amino acid precursor peptide proBNP, it has recently emerged that the release of peptides into the circulation includes not only BNP, but also proBNP and a range of inactive cleavage products of proBNP (Ala-Kopsala et al. 2010; Liang et al. 2007). This has been most intensely studied in acute HF, where most commercial BNP assay kits detect several of the non-BNP peptides (Liang et al. 2007); the extent to which this also occurs in normal subjects is less clear. The molecular analysis of BNP in subjects with acute HF reveals 2 distinct peptides related to: high-molecular weight form, proBNP1-108, and a low-molecular weight form, the biologically active BNP1-32 (Shimizu et al. 2002). There is substantial cross-reactivity of the commercially available BNP and N-terminal pro B-type

natriuretic peptide (NT-proBNP) assays with proBNP (Liang et al. 2007). It appears that proBNP1-108, the intact precursor peptide compared to BNP and NT-proBNP, circulates at high concentrations in patients with HF and may be the predominant form of circulating natriuretic peptide. In addition, it is clear that breakdown products of BNP1-32 circulate as well and most of the degradation fragments of BNP1-32 are also detected by BNP assays. Thus, it appears that there is probably very little bioactive BNP (BNP1-32) in the plasma of HF patients. These findings suggest there are abnormalities in the processing of proBNP1-108 in HF. This problem, along with the breakdown of the BNP1-32 may be contributing to the poor compensatory response of the NP system in chronic HF.

1.6.6 Clinical use of BNP in decompensated CHF

BNP is secreted largely in response to ventricular volume expansion and pressure overload. Muders et al. reported a direct relationship between ventricular wall stress and secretion of BNP (Muders et al. 1997). Tsutamoto and others (Cheng et al. 2001; Tsutamoto, Wada, Maeda, Hisanaga, Fukai, et al. 1997) suggested that BNP is the "emergency hormone" that responds immediately to ventricular overload. In accordance with the physiological effects of BNP, the NPs could be of value clinically in two different aspects: Firstly, their concentrations in the plasma could useful as diagnostic information about cardiac function or structure (de Lemos et al. 2001; Maisel, AS et al. 2002; Rodeheffer 2004; Sun, T, Wang & Zhang 2006). Secondly, NPs have been tried as therapeutic agents in HF (Chen et al. 2012; Dandamudi & Chen 2012; Hobbs, RE et al. 1996; Marcus et al. 1996).

1.6.6.1 BNP and NT-proBNP as a diagnostic modality

In healthy subjects, plasma BNP levels are low, but they rise dramatically in response to cardiovascular stress. Recent studies indicate that increased BNP concentration in plasma

may represent a bio-marker in several cardiovascular diseases, including congestive HF (Gardner et al. 2003; Ruskoaho 2003). It is very important to distinguish HF from other causes of dyspnoea in patients presenting to the emergency department with acute shortness of breath. But sometimes symptoms and physical examination findings are not specific enough to make an accurate diagnosis (Stevenson & Perloff 1989), especially in the presence of pre-existing lung disease. Although echocardiography is considered to be the gold standard for detecting LV dysfunction, it is relatively expensive, not universally accessible and less reliable in the presence of severe pulmonary disease (Devereux, Liebson & Horan 1987). BNP levels have been shown to be elevated in patients with LV dysfunction and correlate with New York Heart Association (NYHA) class as well as prognosis (Clerico et al. 1998; Maeda et al. 1998). A number of studies have shown that elevated BNP levels can predict congestive HF (Clerico et al. 1998; Maeda et al. 1998). Using the Triage® assay (a fluorescence immunoassay), BNP levels can reliably predict the presence or absence of LV dysfunction on echocardiogram (Krishnaswamy et al. 2001). BNP blood concentration measurement is considered to be a sensitive and specific test to diagnose congestive HF in urgent-care settings (Dao et al. 2001). In the setting of acute dyspnoea, Maisel and colleagues (2002) measured BNP in the emergency department, using the Triage® assay, in 1586 patients and compared their results to the final clinical diagnosis. They found that BNP levels were more accurate than any historical or clinical findings or laboratory values in identifying congestive HF as the cause of the dyspnoea (Maisel, A 2002).

1.6.6.2 BNP as a therapeutic tool

With the known cardiorenal and humoral physiological effects, the natriuretic peptide system has been utilized in the treatment of disorders of cardiorenal function including

congestive HF. Two clinical investigations in patients with congestive HF, have demonstrated that acutely administered BNP caused vasodilation, increases in cardiac output and natriuresis in the absence of deleterious neurohumoral activation (Hobbs, RE et al. 1996; Marcus et al. 1996). Intravenous recombinant human BNP (Nesiritide) is becoming increasingly utilized in the therapeutic management of acute decompensated HF recently, where it has favourable actions, largely without adverse effects (Arora, Venkatesh & Molnar 2006; Nishikimi, Maeda & Matsuoka 2006). Studies conducted by Sackner-Bernstein and colleagues raised concerns of potential adverse influence of Nesiritide on renal function and mortality (Sackner-Bernstein, J & Aaronson 2005; Sackner-Bernstein, JD, Skopicki & Aaronson 2005). Furthermore, the ASCEND-HF trial demonstrated that Nesiritide was not associated with reduced rate of death and rehospitalization after 30 days therapy in patients with acute decompensated HF, and Nesiritide therapy did not reduce dyspnea when utilized in combination with standard HF therapies (O'Connor et al. 2011).

1.7 Impaired tissue responsiveness to BNP: how strong is the evidence?

In normal subjects, elevated ANP and BNP levels activate NPR-A, which decreases blood pressure by stimulating natriuresis, diuresis, and vasorelaxation and generally antagonizing the RAAS. Although plasma levels of NPs (as detected by relatively nonspecific commercial assays) rise to very high levels in the setting of acute and chronic HF, recent studies indicated that HF may actually be a state of BNP insufficiency, due to both a deficiency of biologically active BNP 1-32 and resistance to its effects (Chen 2007; Forfia et al. 2007; Hawkridge et al. 2005; Liang et al. 2007). Possible reasons for impaired responsiveness to BNP also include the increased degradation of cGMP (Margulies & Burnett 1994), decreased NPR-A activity (Tsutamoto et al. 1993; Tsutamoto, Wada,

Maeda, Hisanaga, Maeda, et al. 1997) and up-regulated 'clearance' receptors (Andreassi et al. 2001) in patients with HF.

The renal and renin effects of ANP and BNP has been reported to be attenuated in animal models and patients with HF despite marked serum ANP and BNP concentrations (Garcia, Bonhomme & Schiffrin 1992; Supaporn et al. 1996; Tsutamoto et al. 1993). Hawkridge and collegues' (2005) study in severe human HF suggests the absence of circulating BNP1-32 and existence of altered forms of BNP, while another study also reported that BNP8-32 has reduced bioactivity compared with the mature BNP1-32 (Boerrigter et al. 2009).

Furthermore, it has been reported that the reduced NPR-A concentration (Bryan et al. 2007) as well as activity (Dickey et al. 2012; Dickey et al. 2007) in congestive HF is responsible for decreased tissue responsiveness to NPs. Previous studies have suggested that endothelin and AngII released during neurohormonal activation in HF caused the down-regulation of cardiovascular (Gopi et al. 2013; Jaiswal 1992) and renal (Haneda et al. 1991) NPR-A.

In a rabbit model of atherosclerosis, aorta ring relaxation to BNP was attenuated but restored by inhibition of NEP, together with a reduction in atheroma formation. These data suggested the pivotal importance of increased local natriuretic peptide degradation (Schirger et al. 2000).

Moreover, although NPs are thought to inhibit the progression of HF (Munagala, VK, Burnett, JC, Jr. & Redfield, MM 2004), the administration of recombinant BNP (Nesiritide) to HF patients was recently found to be ineffective in acute HF patients (O'Connor et al. 2011). *Therefore, it is potentially of great value to study tissue responsiveness to BNP in*

HF patients to help understand the pathophysiology of the disease and further guide the diagnostic and therapeutic application of BNP.

1.8 Scope of the current study

In the current study, we assessed the possibility that tissue responsiveness to BNP is impaired in patients with acute decompensated HF.

Specifically:

- In section 3.3, we evaluated BNP effects on the neutrophil burst in healthy control subjects.
- In section 3.4, a study demonstrated that BNP effects on the neutrophil burst are impaired in patients with acute HF, despite high circulating BNP levels.
- In section 3.5, it was demonstrated that tako-tsubo cardiomyopathy (TTC) is also associated with tissue desensitization to BNP; this implies that increased BNP release, rather than severe HF, is the basis for desensitization.
- In addition, we explore the possibility that activated inflammation, impaired tissue responsiveness to endogenous or exogenous NO, and increased oxidative stress might play a role in modulating the effects of BNP on neutrophils.

Chapter 2: Methods and materials

2.1 Subject selection:

A total of 50 control subjects with no previously recorded cardiac dysfunction and 45 heart failure (HF) patients admitted to a tertiary care hospital (the Queen Elizabeth Hospital, Adelaide, Australia) with a primary diagnosis of acute HF but without planned coronary revascularization and/or valve replacement were studied. Consistent with international guidelines (McMurray et al. 2012; Yancy et al. 2013), the diagnosis of acute HF was based on the presence of dyspnea at rest or on minimal exertion, together with physical and radiological signs of fluid overload and echocardiographic evidence of systolic and/or diastolic left ventricular dysfunction.

All patients underwent early clinical assessment and simultaneous venesection after (median: 2 days) hospital admission for determination of acute responses to B-type natriuretic peptide (BNP). Following stabilization and at least 3 weeks' (median: 5 weeks) treatment, patients were approached regarding repeat evaluation (n=25).

The study was approved by the Queen Elizabeth Hospital Ethics of Human Research Committee and informed consent was obtained prior to study entry.

2.2 Blood sampling

Blood samples were drawn by venesection from an antecubital vein. Blood was collected into heparinized vacutainer tube for whole blood reactive oxygen species (ROS) assessment; a tube containing 24mmol/L EDTA for neutrophil preparation. For platelet aggregometry, blood was collected in plastic tubes containing 1:10 volume of acid citrate anticoagulant (two parts of 0.1 mol/L citric acid to three parts of 0.1 mol/L trisodium

citrate). Acidified citrate was utilized to minimize deterioration of platelet function during experiments (Kinlough-Rathbone, Packham & Mustard 1983).

2.3 Preparation of neutrophils

For neutrophil preparation, blood samples were centrifuged at 150*g* for 10 minutes, and plasma was replaced by an equal volume of Hanks' balanced salt solution (HBSS), pH 7.4. Neutrophils were isolated using a Ficoll-Hypaque gradient centrifugation as previously described (Boyum 1968). Following centrifugation at 550*g* for 30 minutes, the lower layer containing neutrophils and red blood cells was collected. Following lysis of the red blood cells and washing to remove the lysis buffer (ammonium chloride 155 mmol/L, EDTA 100 mol/L, $NaHCO_3$ 10 mmol/L, pH 7.4), the neutrophils were pelleted (550*g*, 10 minutes) and resuspended in HBSS (pH 7.4) at $1.7x10^6$ cells per mL. The viability of neutrophils was shown to be over 95% by Trypan blue exclusion.

2.4 Intra-neutrophil cGMP determination

2.4.1 Methodological experiments

Experiment 1. Determination of optimal neutrophil cell concentration and BNP incubation time.

Intra-neutrophil cGMP content was assayed according to the previously described methodology for intra-platelet cGMP content analysis (Chirkov et al. 1999). Briefly, neutrophils suspensions (1mL) at concentration of 2×10^6, 5×10^6, 10×10^6, 20×10^6 cells/mL were pre-incubated at 37°C with BNP (1μmol/L) (Huntley et al. 2006) for 1, 5, 10 minutes in the presence of the phosphodiesterase inhibitor 3-Isobutyl-1-methylxanthine (IBMX, 1mmol/L, added to the sample 1 minutes before BNP). Samples were mixed every 3

minutes during incubation. After incubation, samples were filtered through GF/C Glass Microfibre Filters (Whatman) to harvest the neutrophils. Filters with neutrophils were rinsed with saline (0.9%) and placed into the glass tubes containing 0.5 mL EDTA (4mmol/L). After boiling in a water bath for 5 minutes, tubes were centrifuged at 3000*g* for 10mintes and cGMP concentration in the supernatant was estimated using "cGMP [I^{125}] assay system" (Biomedical Technologies Inc, Soughton, MA, USA). A representative standard curve is shown in Figure 2-1, and results are shown in Table 2-1.

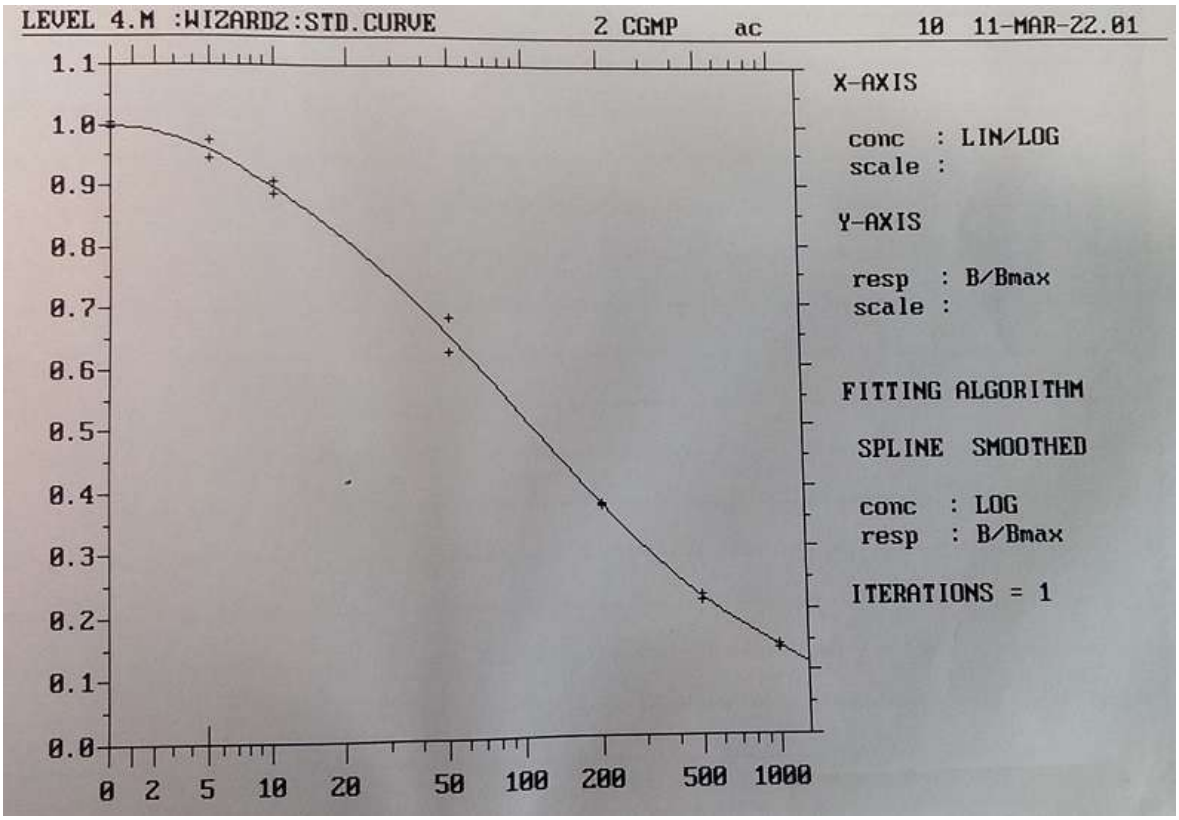

Figure 2-1: Representative standard curve for cGMP [I^{125}] radioimmunoassay.

Table 2-1: Impact of duration of incubation with BNP on changes in intra-neutrophil cGMP content (fmol).

Experiment NO.	Vehicle	IBMX	IBMX BNP 1min	IBMX BNP 5min	IBMX BNP 10min	cells/mL
1	10.96	11.00	9.87	11.14	12.84	2×10^6
2	14.03	10.51	10.51	9.51	8.74	5×10^6
3	24.42	17.01	13.86	14.38	15.08	5×10^6
4	10.75	17.46	23.35	34.26	42.67	10×10^6
5	10.81	13.95	9.78	9.77	11.98	10×10^6

Results of this set of experiments showed that 10 minutes incubation with BNP and 10×10^6 cells/mL was superior to other conditions. However, the signal was low: within the 5 experiments there was no statistically significant increase in intro-neutrophil cGMP content with different cell numbers and incubation times. The reasons might include:

(1). a proportion of cells might be damaged during filtering and rinsing

(2). The EDTA solution (4mmol/L) might be not strong enough to fragment the cells;

(3). Interference between the buffer and samples might exist;

(4). Boling the samples might not be sufficient to release all the cGMP.

Therefore, the following experiments were designed to address these concerns.

Experiment 2. Determination of cGMP in neutrophils: no filtering.

In this set of experiment, the nitric oxide (NO) donor sodium nitroprusside (SNP) was applied. SNP is known to activate sGC and induce cGMP formation. Samples (0.5 mL with 20×10^6 cells/mL) were incubated with/without BNP/SNP for 5minutes, and at the end of the incubation 0.5 mL EDTA was added directly into the sample and boiled for 5 minutes. Same cGMP assay methodology was applied as in experiment 1. Results (Table 2-2) revealed similar values as obtained in experiment 1 (Table 2-1), and indicated that the filtering of neutrophils was not the reason for the low cGMP detection.

Table 2-2: Results of experiment 2 (no neutrophil filtering).

Vehicle (fmol)	IBMX (fmol)	IBMX+BNP 5min (fmol)	IBMX+SNP 5min (fmol)	cells/mL
16.51	15.56	14.81	15.08	20×106

Experiment 3. Determination of cGMP in neutrophils: no filtering and hydrochloric acid instead of EDTA: effects of incubation with SNP.

In this set of experiments, samples (0.5 mL with 20×10^6 cells/mL) were incubated with/without SNP (rather than BNP) for 1minutes, and at the end of the incubation 0.5 mL ice cold hydrochloric acid (HCl 0.1mol/L) was either added directly into the samples or samples were pelleted by centrifugation (550*g* for 10 minutes at 4ºC) and cells were re-suspended in 0.5 mL of HCl (0.1mol/L). After that, all of the samples were boiled for 5 minutes. The same radioimmunoassay assay method was applied as in experiment 1. Results are shown in Table 2-3. Although the protein extraction methods utilized for these

two sets of sample were different, the results were similar. However, the second set of experiments was better because the values were on the more accurate area of the standard curve (Figure 2-1), and it was possible to detect cGMP.

Table 2-3: Results of experiment 3.

Experiment NO.	Vehicle	IBMX	IBMX+SNP 1min	cells/mL
1	14.81	15.69	18.08	10×10^6
2	29.71	33.33	39.88	20×10^6

Experiment 4. Determination of cGMP in neutrophils after sonication: impact of SNP.

In this set of experiments, neutrophil samples (0.5 mL, 20×10^6 cells/mL) were incubated with/without SNP for 1minute, and at the end of the incubation samples were pelleted by centrifugation (550*g,* 10 minutes at 4 ºC) and cells were re-suspended in 0.5 mL HCl (0.1mol/L). After that, samples were boiled for 5 minutes and underwent sonication (sonicator bath [Unisonics PTY.LTD, NSW, Australia] or Branson Sonifer-250 [Danbury, CT, USA]) to further break down the cells. Same radioimmunoassay assay method applied as in experiment 1. Results are shown in Table 2-4. These results indicated that the effect of these different sonication methods were similar.

Table 2-4: Results of experiment 4.

Incubation reagent	Sonication methods	cGMP content (fmol)
Vehicle	Unisonics	16.51
	Branson Sonifer	16.83
IBMX	Unisonics	25.26
	Branson Sonifer	17.53
IBMX+SNP	Unisonics	20.87
	Branson Sonifer	19.45

Experiment 5. Determination of cGMP in neutrophils: different cGMP extraction conditions.

In this set of experiments, after incubation of neutrophils with SNP, either (1) HCl (0.1mol/L), (2) sodium acetate buffer (0.05mol/L, pH 6.2, as used in the cGMP radioimmunoassay kit), (3) HBSS (pH 7.4, the same buffer used for neutrophil suspension) was added to the samples. All samples were then boiled in a water bath for 5 minutes. After centrifugation of samples at 2500*g* for 10 minutes, cGMP concentration in supernatant was estimated using the same radioimmunoassay assay method applied in experiment 1. Results are shown in Table 2-5.

The results in this experiment showed that the acetate buffer should be used in this assay and interference existed between sample and antibody supplied by the manufacture. Therefore, experiments were designed to clarify this interference.

Table 2-5: Results of experiment 5: generation of cGMP in neutrophils on incubation with SNP.

Solutions/buffers added after incubation	Incubation reagent	cGMP content (fmol)
HCl	Vehicle	19.55
	IBMX	24.43
	IBMX+SNP	24.05
Acetate buffer	Vehicle	31.44
	IBMX	37.07
	IBMX+SNP	23.11
HBSS buffer	Vehicle	206.04
	IBMX	179.54
	IBMX+SNP	136.85

Experiment 6. Determination of the interfering substrate on cGMP assay in neutrophils:

In this set of experiments, the samples (with no buffer added) collected from the last experiment were assayed, extra amount of cGMP standard/normal saline (NaCl) was added into each sample. Sample combinations and results are shown in Table 2-6. The results of this experiment showed the extra amount of 25fmol cGMP standard was quenched by the original sample and confirmed the interference claimed in the last experiment.

Table 2-6: Sample preparations and results for experiment six.

Samples with HBSS buffer only	cGMP content (fmol)
cGMP standard 25fmol (50µL) + NaCl (50ul)	24.35
Vehicle (100µL)	206.04
Vehicle (50µL) + cGMP standard 25fmol (50µL)	89.61
Vehicle (50µL) + NaCl (50µL)	99.58
IBMX+SNP (100µL)	136.85
IBMX+SNP (50µL) + cGMP standard 25fmol (50µL)	68.27
IBMX+SNP (50µL) + NaCl(50µL)	69.79

Samples in red indicate that the values were obtained from the last experiment.

Experiment 7. Sample purification before cGMP assay:

Samples (with no buffer added) collected from experiment 5 were assayed. Samples were centrifuged at room temperature for 5 minutes at 3000*g* and purified by solid phase extraction using vacuum manifold-10 columns and Strata SAX cartridges (Phenomenex, CA, USA). The solid phase extraction cartridges were equilibrated with methanol and water before 250µL samples were transferred into the columns. After all the solutions passed through the columns, 3mL methanol was passed through the column under vacuum and collected, and then 3mL acidified methanol (0.1mol/L HCl in methanol) was passed through the column and collected as well. The samples were then evaporated under the

flow of nitrogen at 37°C and reconstituted in 250µL acetate buffer (0.05mol/L, pH 6.2). Samples were assayed as shown in Table 2-7.

Results of this experiment indicated that HBSS interferes with the cGMP assay. The recovery of the additional 25fmol cGMP standard was approximately 100% after samples been extracted. And dilution of sample (Sample B) did not improve the accuracy of the results. Therefore, it was necessary to clean up the samples by extraction after incubation.

Table 2-7: Sample conditions and results for experiment 7: effects of HBSS buffer.

Samples	Samples' conditions	cGMP content (fmol)
HBSS	HBSS buffer alone (100µL)	11.86
Sample A	original (100µL)	57.97
	extracted (100µL)	47.14
	extracted (100µL) + 25fmol (cGMP standard)	73.64
Sample B	original (50µL) + Acetate buffer (50µL)	32.20
	original (25µL) + Acetate buffer (75µL)	23.43
	original (12.5µL) + Acetate buffer (87.5µL)	22.36

2.4.2 Final experiment protocol for intra-neutrophil cGMP determination:

Neutrophil suspensions (500µL) were pre-incubated at 37°C with either BNP (1µmol/L) for 10 minutes or SNP (10µmol/L) for 1 minute in the presence of the IBMX (1mmol/L).

Intra-neutrophil cGMP content was assayed via radioimmunoassay. After incubation, samples were placed on ice and centrifuged at 550*g* for 5 minutes at 4°C. Pellets were re-suspended in 500μL ice cold acetate buffer then put in a boiling water bath for 5 minutes and sonicated for 5 minutes using the sonication bath (Unisonics PTY.LTD, NSW, Australia). After centrifugation of samples at 2500g for 10 minutes, cGMP concentration in supernatant was extracted and estimated using "cGMP [I^{125}] assay system" (Biomedical Technologies Inc, Soughton, MA, USA). Results were expressed as increase (%) in intra-neutrophil cGMP accumulation in response to BNP or SNP in the presence of IBMX in comparison with IBMX alone.

2.5 Electron paramagnetic resonance (EPR) spectroscopy measurement of ROS

2.5.1 Theory of EPR spectroscopy

Electron paramagnetic resonance (EPR), also called electron spin resonance (ESR) is a technique which quantitates free radicals and transition metal ions with unpaired electrons by absorption of microwave radiation stimulated by free radicals in an electromagnetic field (Frejaville et al. 1995; Zhang et al. 2000). The components of an EPR spectrometer includes a microwave generator, a resonator cavity centered between a pair of electrical magnets and a recorder of the translated signal intensity (Figure 2-2 A). The microwaves generated by the microwave generator are transmitted to the resonator cavity, and after adjustment of the spectrometer, all of the energy will be absorbed which results in no detection of microwave energy by the resonator. When the sample is placed into the magnetic field, the unpaired electrons will orient to the same direction as the magnetic field, which generates 2 different energy states for the unpaired electrons and a transition

from the lower state to the higher state will be formed by absorption of microwave energy (Figure 2-2 B) (Weil, Bolton & Wertz 1994). The different electron energy formed between the 2 states is equivalent to the microwave energy ($h\upsilon=g\times\mu_B\times B$, "$h\upsilon$" is the microwave energy, "g" is the factor constant equal to 2.002 for most organic samples, and "μ_B" is the Bohr magneton constant) (Dikalov, S, Griendling & Harrison 2007).

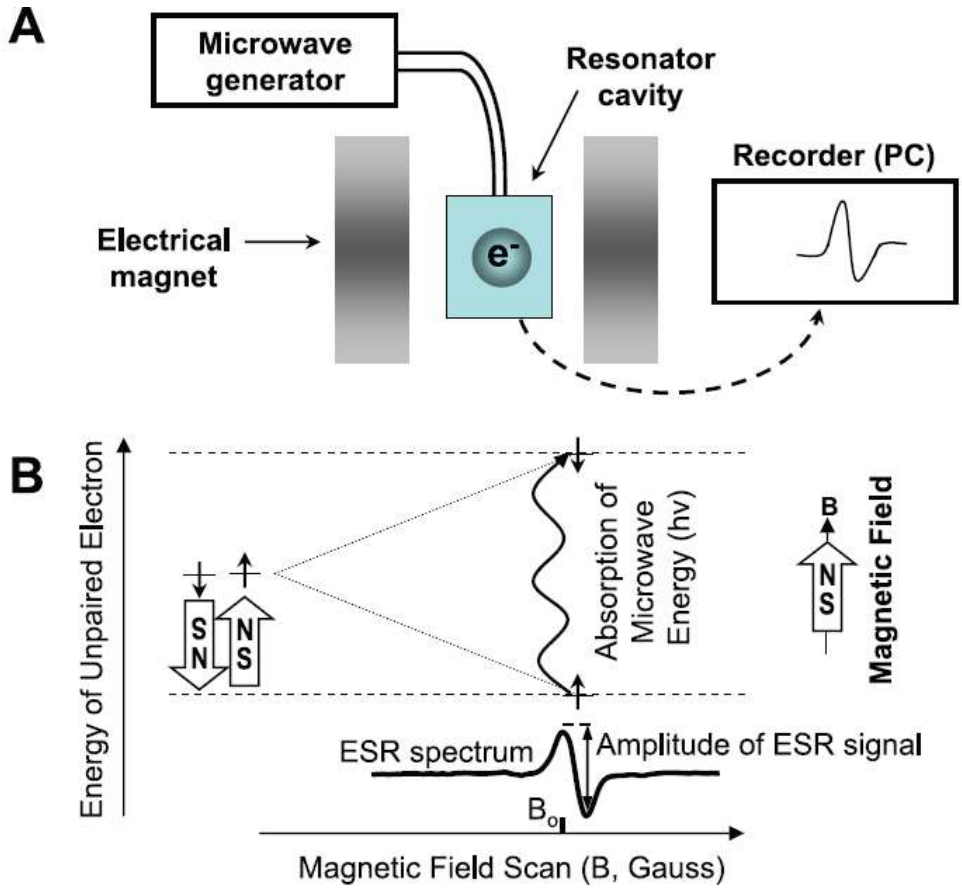

Figure 2-2: The typical EPR spectrometer

(A) EPR composition. The electron has a magnetic moment; it acts like a compass when it placed in a magnetic field (B). It will have a state of lowest energy when the moment of the electron is aligned with the magnetic field (↑) and a state of highest energy when it aligned against the magnetic field (↓). The energy of the unpaired electron, therefore, is a function of the magnetic field: $E=\pm 0.5g\mu_B B$, where "g" is the g-factor constant, "μ_B" is the Bohr

magneton constant, and "B" is the magnetic field. Adapted from: (Dikalov, S, Griendling & Harrison 2007)

In summary, the transition formed by placing the sample containing unpaired electrons in an applied magnetic field will be detected by EPR as absorption of microwave energy. The amplitude of the EPR signal (microwave energy) represents the number of the unpaired electrons present in the sample (Figure 2-2 B), thus enable quantification of free radicals. The EPR spin-trapping technique has been used to detect ROS in whole blood (Fink, Dikalov & Bassenge 2000) and O_2^- generation in neutrophils induced by inflammation via NAD(P)H oxidase in cellular systems *in vitro* (Bannister et al. 1982; Dikalov, SI et al. 2011; Tanigawa, Kotake & Reinke 1993).

The half-life of most free radicals is very short and makes it a challenge to detect them in biological samples. A variety of chemicals that form stable adducts with free radicals have been studied and developed as "spin traps" (Janzen 1984), and used for detection of free radicals (Dikalov, S, Jiang & Mason 2005; Dikalova, Kadiiska & Mason 2001; Zhang et al. 2000). However, it was found that the nitrone radical adducts are not stable. The O_2^- adduct EMPO-OOH (trapped by EMPO) is rapidly converts to EMPO-OH, which are identical to OH• radical adducts, by GPx within seconds (Zwicker et al. 1998). Therefore, it is difficult to distinguish one from another without a specific inhibitor. Also, in biological samples, nitrone radical adducts can be reduced to EPR silent species by a number of compounds such as flavins, thiols and ascorbate etc. (Figure 2-3). Furthermore, the reactivity of nitrone spin traps with O_2^- is relatively low compared to cytochrome c (Koppenol et al. 1976). Therefore, investigators have been focused on developing of more effective and specific spin probes.

The cyclic hydroxylamines, although not spin traps, can be oxidized by free radicals to form stable radicals with much longer half-lives (up to several hours). They have been proven to be effective and easily be detected by EPR in tissues and cells (Dikalov, S, Skatchkov & Bassenge 1997) for detection and quantitation of O_2^- both extra- & intra-cellular as well as mitochondrial origin and showed more effective than the nitrone spin traps, for review see (Dikalov, SI et al. 2011). Furthermore, the formation of nitroxide by hydroxylamine is stable and persistent with reductase in biological samples (Figure 2-3 B) (Dikalov, S et al. 1997).

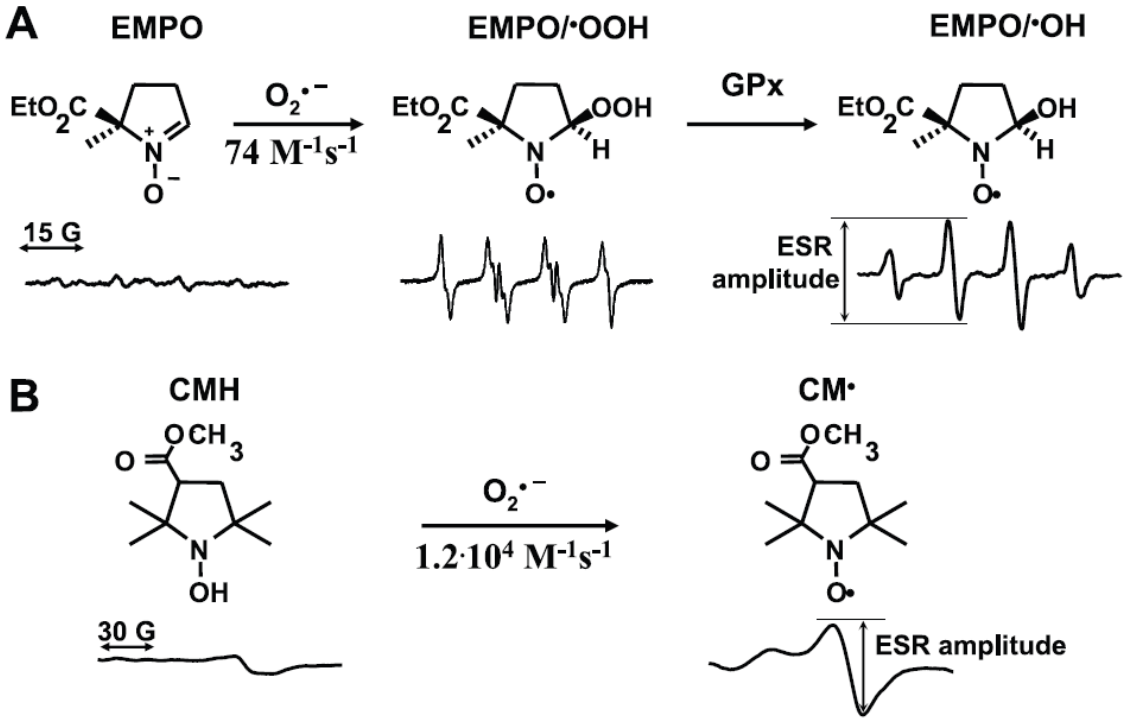

Figure 2-3: Detection of O_2^- by ESR.

A, Spin trapping of O_2^- by spin trap EMPO produces O_2^- radical adduct EMPO/OOH, which is decomposed by glutathione peroxidase (GPx) into OH-adduct and reduced by flavin enzymes and ascorbate into ESR silent hydroxylamine. B, The reaction of O_2^- with

spin probe CM-H produces stable nitroxide radical, which can be conveniently detected in frozen samples. (Dikalov, S, Griendling & Harrison 2007).

Detection of intracellular O_2^- by EPR showed that CM-H is highly cell permeable with the highest rate of intracellular accumulation of nitroxide among all the tested hydroxylamines (Figure 2-4 and Figure 2-5). However, as hydroxylamines can be oxidized by several ROS, it is important to perform experiments with SOD, ONOO- scavengers, or other inhibitors to identify which ROS generated the signal in individual case.

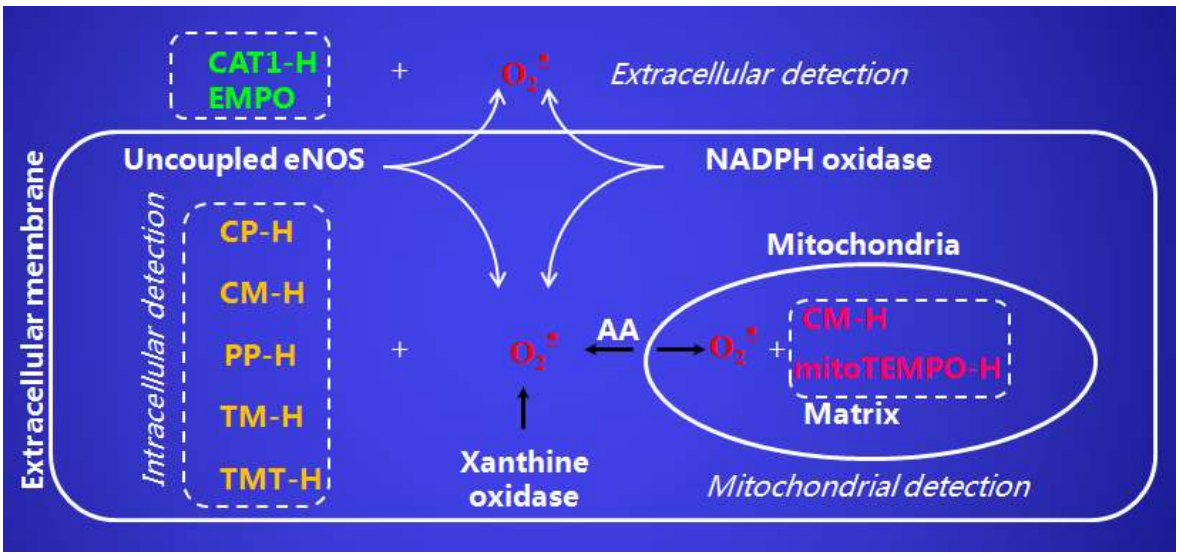

Figure 2-4: General scheme of EPR detection of O_2^- in extracellular, intracellular or mitochondrial compartments using cyclic hydroxylamine spin probes.

Cyclic hydroxylamines 1-hydroxy-2,2,6,6-tetramethylpiperidin-4-yl-trimethylammonium chloride (CAT1-H), 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine hydrochloride (CP-H), 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl pyrrolidine (CM-H), 1-hydroxy-4-phosphono-oxy-2,2,6,6-tetramethylpiperidine (PP-H), 1-hydroxy-4-methoxy-2,2,6,6-tetramethylpiperidine (TM-H), N-(1-Hydroxy-2,2,6,6-tetramethylpiperidin-4-yl)-2-

methylpropanamide (TMT-H), 1-hydroxy-4-[2-(triphenylphosphonio)-acetamido]-2,2,6,6-tetramethylpiperidine (mitoTEMPO-H,), spin trap 5-ethoxycarbonyl-5-methyl-1-pyrroline N-oxide (EMPO). Adapted from: (Dikalov, SI et al. 2011).

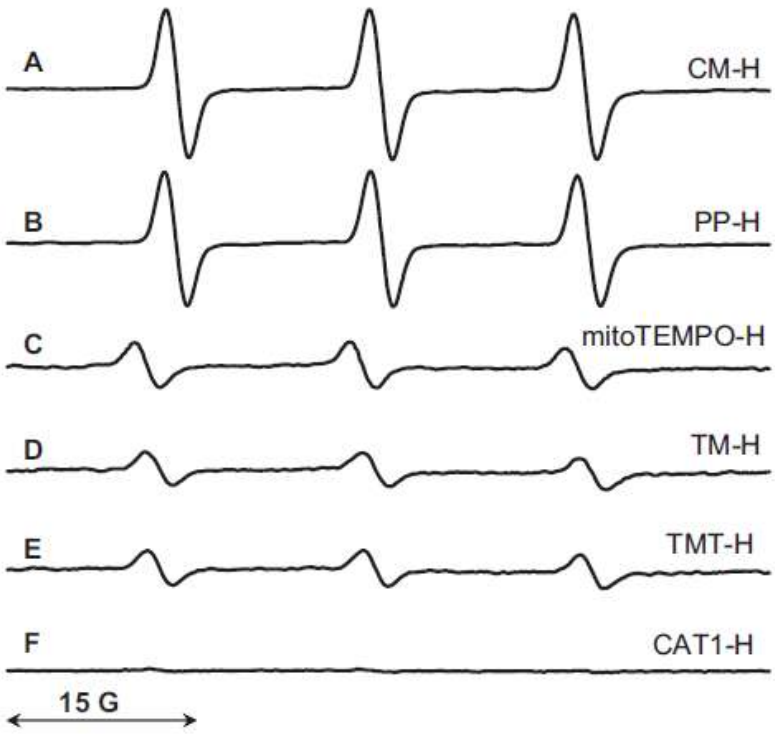

Figure 2-5: Representative EPR signals captured by hydroxylamine spin probes with different Cell permeability.

This figure demonstrated that CM-H is the most cell-permeable spin probe among those tested. Data were obtained in confluent rat aortic smooth muscle cells. Adapted from: (Dikalov, SI et al. 2011).

2.5.2 Whole blood ROS determination by EPR spectroscopy

Quantitation of total ROS in whole blood was performed utilizing EPR spectroscopy, as previously described (Mariappan et al. 2009; Mrakic-Sposta et al. 2012). Whole blood samples were incubated with BNP (1μmol/L) or vehicle for 10minutes before measurement by EPR spectroscopy. All EPR samples were prepared using the spin probe CM-H (0.2mmol/L) in Krebs - Hepes buffer (pH 7.4) in the present of 25μmol/L deferoxamine and 5μmol/L sodium diethyldithiocarbamate trihydrate and placed in 50μL Micropipettes DURAN® glass capillaries (Noxygen, Elzach, Germany). Reaction of ROS with CM-H generates a stable nitroxide radical, the formation of which was measured by monitoring the amplitude of the low-field component of the EPR spectrum as previously described (Rosen, Finkelstein & Rauckman 1982), and calculated from the accumulation of nitroxide, obtained from a calibration curve for intensity of the EPR signal of 3-carboxyproxyl. Total ROS formation in blood was determined from the time-dependent accumulation of the stable nitroxide radical. For this purpose, EPR kinetics were analysed by linear regression using WinEPR software (Bruker Biospin Corp, Billerica, MA, USA).

EPR spectra were recorded using an e-scan M EPR spectrometer (Bruker, Germany, Figure 2-6) and super-high Q microwave cavity with the following settings: field sweep, 10 G; microwave frequency, 9.75 GHz; microwave power, 19 mW; modulation amplitude, 2 G; conversion time, 10.24 ms; time constant, 40.96 ms; receiver gain, 3.2×10^2. Data were expressed as μM/mL/minutes of nitroxide accumulation. Intra-assay CV was 5.6%.

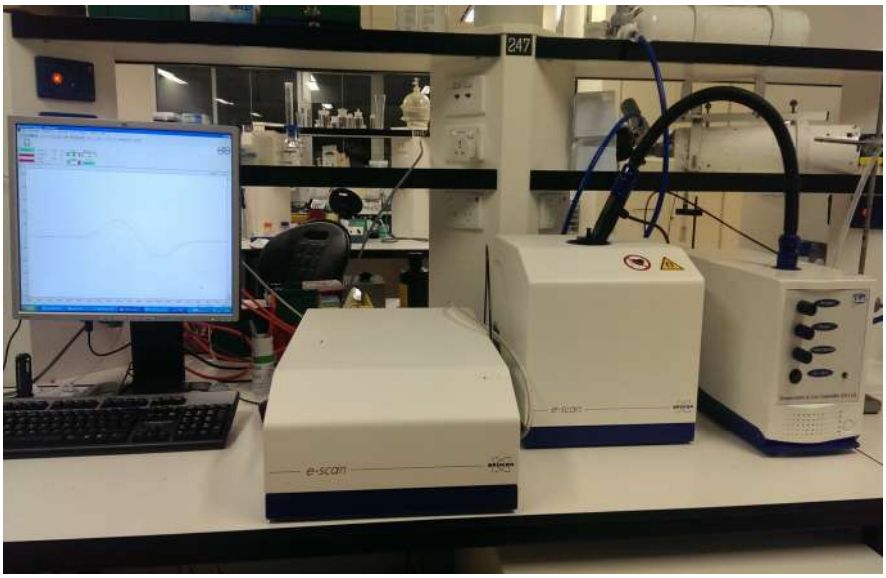

Figure 2-6: Electron paramagnetic resonance (EPR) spectroscopy (Bruker BioSpin e-scan).

From left to right: PC recorder, microwave generator, magnetic unit and temperature and gas controller BIO-III.

2.5.3 Determination of O_2^- generation in isolated neutrophils by EPR spectroscopy

Neutrophil intracellular O_2^- accumulation was determined by EPR as previously described (Dikalov, SI, Dikalova & Mason 2002; Dikalov, SI et al. 2011). Specificity of O_2^- detection by CM-H was validated by supplementation of SOD which eliminated approximately the entire EPR signal (Figure 2-7) (Dikalov, SI et al. 2011). In the current study, neutrophils were incubated with either BNP (1μmol/L, 100nmol/L, 10nmol/L, and 1nmol/L) or 8-(4-Chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate (8-pCPT-cGMP, 500μmol/L) for 10 minutes then stimulated with either phorbol 12-myristate 13-acetate (PMA, 100nmol/L, for 20minutes) or *N*-formyl-methionyl-leucyl-phenylalanine (fMLP, 1μmol/L) (Liu, FC et al. 2012) before the addition of CM-H (0.2mmol/L). Samples

were scanned immediately after supplementation of spin probe CM-H. As the EPR signal is a measure of the total ROS, for assessment of the O_2^- component, values obtained in presence of the NAD(P)H oxidase inhibitor DPI (18µmol/L), and results showed that DPI inhibited all the PMA-induced O_2^- release (Figure 3-2). EPR settings were identical to those utilized for whole blood ROS determination. EPR experiments were performed in triplicate.

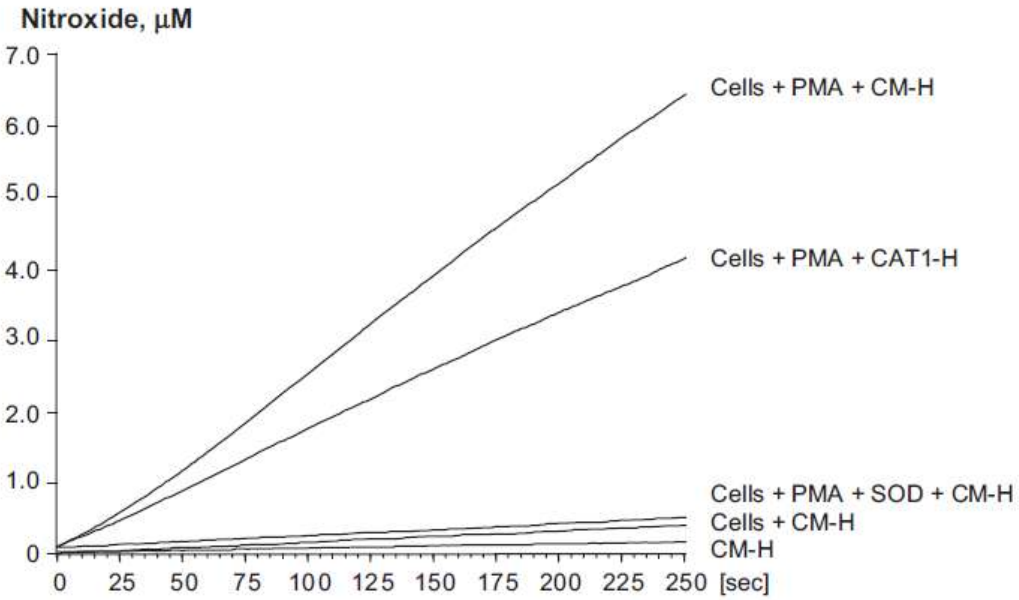

Figure 2-7: O_2^- production by PMA-stimulated human neutrophils.

Cells were stimulated by 1µmol/L PMA with/without SOD and O_2^- was measured with 1µmol/L spin probes. Adapted from: (Dikalov, SI et al. 2011).

2.6 Western blotting analysis

2.6.1 Sample preparation

Isolated neutrophils from control subjects and HF patients were pre-treated with or without BNP (1μmol/L) for 10 minutes at room temperature and then stimulated with fMLP for 20 minutes. The reaction was stopped by placing samples on ice. After centrifugation (550*g* for 10 minutes at 4°C) the cells were lysed by resuspension in lysis buffer (Tris-HCl 100mmol/L, NaCl 0.45mol/L, EDTA 50mmol/L, EGTA 10mmol/L, sodium pyrophosphate 20mmol/L, β-glycerophosphate 20mmol/L, protease inhibitor cocktail 1, 1%, protease inhibitor cocktail 2, 1%, phosphatase inhibitor, 1%, Triton X-100 10%). Proteins in the cleared supernatant were denatured in Laemmli's sample buffer (with 5% 2-mercaptoethanol). The samples were then subjected to SDS (10%) -PAGE and western blot using standard techniques. The separated proteins were transferred to nitrocellulose membrane, which was blocked with 5% milk/ bovine serum albumin in TBS containing Tween 20 (0.1%) for 1hour. After blotting, the membranes were probed with the appropriate primary antibody and followed by incubation with HRP-labeled secondary antibody accordingly.

2.6.2 Immunoblot analysis of p47phox and phosphorylation of p47phox in neutrophils

The phosphorylated-p47phox at Ser345 and total p47phox was bloted on separate gels. The primary antibody dilutions were as follows: p47phox (D21F6) rabbit monoclonal antibody (1:1000) (Cell Signaling Technology, Danvers, Massachusetts, USA), rabbit polyclonal to p47phox (Phospho-Ser345) (1:1000) (Biorbyt Limited, Cambridge, UK) and Mouse anti β-actin monoclonal antibody (1:2500) (Abcam, Cambridge, UK).The intensity of

phosphorylated-p47phox at Ser345, total p47phox and β-actin on the same membrane was visualized by chemiluminescence using ImageQuant LAS-400 (GE Healthcare Life Sciences, Buckingham, UK) and quantified by densitometry using the Multi Gauge V3.0 analysis program. Phosphorylated intensities were corrected for the corresponding amounts of total p47phox present on the membrane. Because the p47phox and phosphor p47phox Ser345 has the same molecular weight (47kD), the two proteins were blotted on separate membranes, results were correlated with β-actin presented on each membrane. Example immunoblots are shown in Figure 2-8.

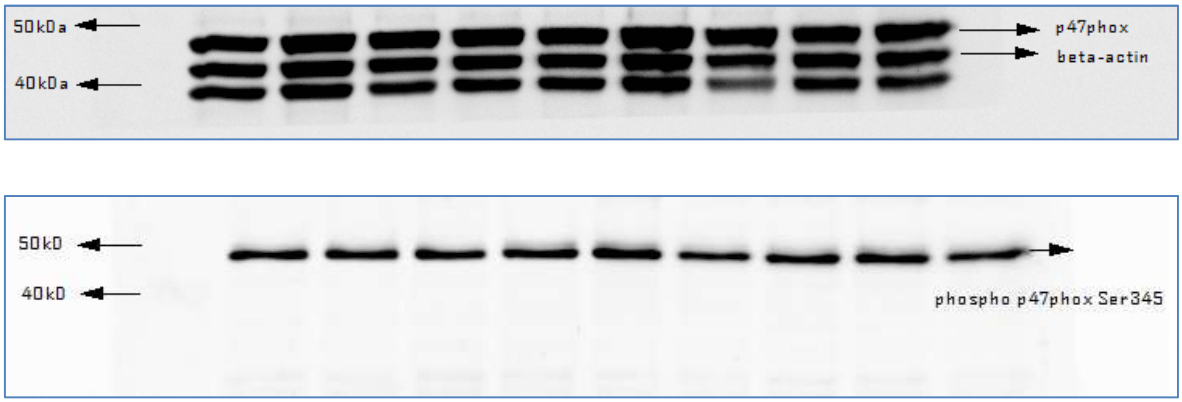

Figure 2-8: Example blots showing p47phox, β-actin (upper panel) and phospho-p47phox Ser345 (lower panel).

2.6.3 Immunoblot analysis of NPR-A/pGC-A in neutrophils

For NPR-A blots, rabbit anti-PGC-A rabbit polyclonol antibody was probed in untreated neutrophil lysate. Several primary antibody dilutions were attempted, including 1:500, 1:1000, 1:2000 and 1:5000 in combination with secondary antibody dilutions: 1:500,

1:1000, 1:2000 and 1:5000. However, no visible protein band was revealed (no results will be shown for this part).

2.7 Assessment of endothelial function

2.7.1 Applanation tonometry

Pulse wave analysis, together with administration of endothelium-independent vasodilator NTG and endothelium-dependent vasodilator salbutamol (β_2-agonist), was used to evaluate vascular NO responsiveness and endothelial function, respectively.

Augmentation index (AIx), as a measure of arterial stiffness was determined using SphygmaCor device (AtCor Medical, Sydney, Australia), as previously described (Hayward et al. 2002; Wilkinson et al. 2002). A high-fidelity micro-manometer probe (SPT-301B; Millar Instruments, Texas, USA) was used to obtain recordings of the peripheral pressure waveforms by flattening, but not occluding, the radial artery of the dominant arm at the position where the pulse was most evident. Radial artery pulse waveforms were recorded directly onto a computer-linked SphygmaCor analysis system (SCOR-Px, software version 7.01, AtCor Medical Pty Ltd. Sydney, Australia). The SphygmaCor system generated an average peripheral pulse waveform contour from an 11-second recording period (Figure 2-9). The corresponding central (ascending aortic) waveform was derived from the radial artery waveform using a validated mathematical transfer function within the software package (SphygmaCor). From this a rate corrected augmentation index, a value of change in AIx, which is a measure of arterial stiffness, was derived and corrected for a standard heart rate of 75 bpm. Representative waveforms are shown in Figure 2-9.

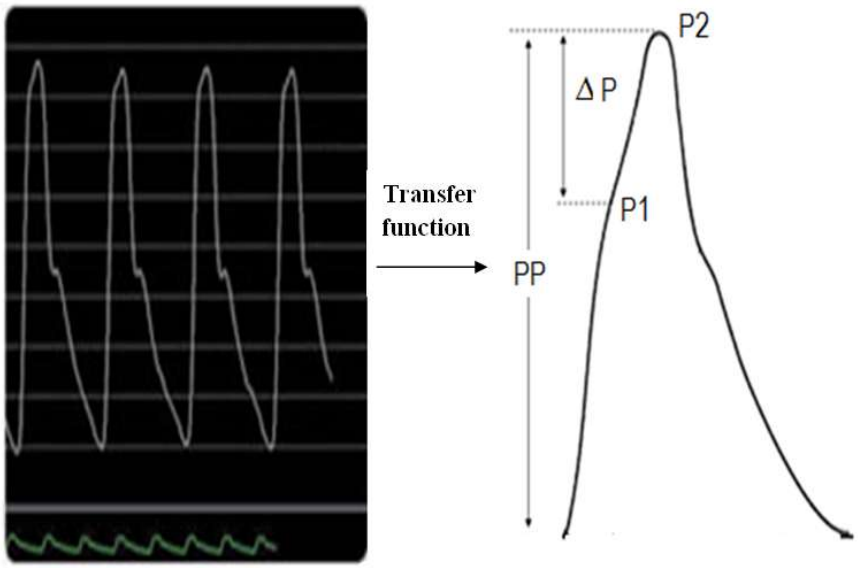

Figure 2-9: Pulse wave analysis, representative waveforms.

Peripheral pressure waveforms recorded electronically with the SphygmaCor device. The peripheral waveform is converted to a central waveform via an integral transfer function to derive a rate-corrected augmentation index. AIx is the difference (ΔP) between the first (P1) and second systolic (P2) pressure peaks, divided by pulse pressure (PP), expressed as a percentage of the pulse pressure, which is used to evaluate vascular endothelial function and NO responsiveness profiles. Adapted from: (Crilly et al. 2007)).

Baseline pulse recordings were taken in triplicate and averaged. After obtaining baseline augmentation index values, subjects were administered sublingual NTG (50 µg). Blood pressure was measured at 2 minutes intervals for 20 minutes. NTG produces generalized vasodilation, thereby decreases venous return and workload on the heart. Previous studies have confirmed that 20 minutes was sufficient for haemodynamic changes to return to baseline after administration of NTG (Wilkinson et al. 2002). After augmentation index had returned to baseline values, the protocol was repeated for salbutamol (400 µg),

administered by inhalation with a spacer device. Immediately after each blood pressure measurement, radial pulse recordings were taken. The arterial pressure waveforms were calibrated with the brachial diastolic and systolic pressure and a calibration system integral to the device. Only high quality recordings with an in-device quality index ≥ 85 % were used. Effects of NTG and salbutamol were quantified by determination of the drug-induced changes in rate adjusted augmentation index. Vascular responses to NTG and salbutamol were expressed as area under the curve in augmentation index. As shown in Figure 2-10, subjects with endothelial dysfunction have a decreased response or do not respond to endothelial dependent vasodilator, salbutamol. Subjects with NO resistance have a diminished response to NTG.

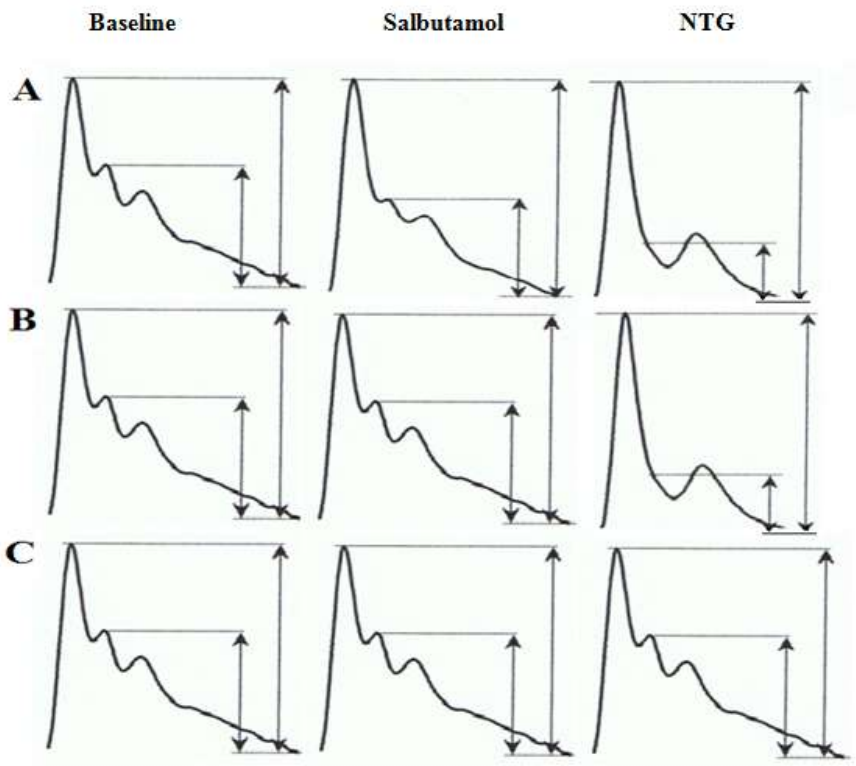

Figure 2-10: Effects of salbutamol and nitroglycerin (NTG) on the radial waveforms derived from pulse wave analysis.

Panel A: *Normal Subject: The second systolic peak, at baseline, is diminished by salbutamol and further decreased following NTG. The change in the wave-shape is quantified using AIx.*

Panel B: *Subject with endothelial dysfunction: no response to endothelium-dependent NO donors.*

Panel C: *Subject with impaired vascular NO responsiveness: no response to endothelium-dependent and -independent NO donors.*

Adapted from: (Hayward et al. 2002).

2.7.2 Determination of Plasma ADMA, Symmetric Dimethylarginine and L-arginine

Plasma concentrations of the endogenous NOS inhibitor ADMA, Symmetric Dimethylarginine (SDMA) and L-arginine were determined via high-performance liquid chromatography as previously described (Heresztyn, Worthley & Horowitz 2004). Blood was collected into heparinised tubes, placed on ice immediately and then centrifuged at 1800*g* for 15 minutes at 4ºC. The plasma was collected and stored at -80ºC until assayed.

2.7.2.1 Sample Extraction and Derivatization

Plasma samples were defrosted and vortexed to make solution homogeneous. Samples were then centrifuged at 1800*g* for 10 minutes. All samples were diluted by adding 150µL of plasma sample to 1.4mL of distilled H_2O, and 60µL of 5µg/mL N-monomethyl-L-arginine (L-NMMA) which was treated as an internal control. 300µL 10% (w/v) 5-sulfosalicyclic acid was added into the solution to precipitate plasma proteins from

solution and incubated on ice for 10 minutes. Samples were then centrifuged at 9000*g* for 2 minutes at room temperature and the supernatant was retained. Samples underwent solid phase extraction using a Gilson GX-274 ASPEC Liquid Handler (run using Trilution LH version 2.0 software, Gilson) and Bond Elut SCX cartridges (Agilent Technologies, US). The solid phase extraction cartridges were washed with 0.1M phosphate buffer, pH 6.0 and methanol prior to eluting the analytes with 2% (w/v) triethylamine/65% (v/v) methanol in distilled H_2O. The eluent was evaporated under nitrogen flow at 55ºC and reconstituted in distilled H_2O. Samples were centrifuged at 9000*g* for 2 minutes at room temperature and 50µL supernatant transferred into fresh vials to be derivatized using the AccQ-Fluor Reagent Kit (Waters, UK).

2.7.2.2 Chromatographic Separation and Fluorescent Detection

Samples were loaded onto an 1100 series HPLC system (Agilent Technologies, US) with a 1200 series fluorescence detector (Agilent Technologies, US) using a 717plus Autosampler (Waters, UK) maintained at 12ºC and the analytes separated on a Luna 5µm C18(2) column (Phenomenex, US) using a gradient of 4% (v/v) acetonitrile in 0.1M sodium acetate, pH 6.0 (Mobile Phase A) and 30% (v/v) acetonitrile in 0.1M sodium acetate, pH 6.0 (Mobile Phase B) at a flow rate of 1.0mL/minute. Column temperature was maintained at 40°C using a TCM-004055 incubator (Waters, UK) for ADMA/SDMA (20µL injection volume) determination and 30°C for L-arginine (2.5µL injection volume) determination. Analyses were performed separately for ADMA/SDMA and L-arginine determinations. Fluorescent detection of derivatized sample was achieved using excitation at λ=250nm and emission at λ=395nm. The system was managed using ChemStation for LC 3D Systems software, version Rev B.03.02 [341].

2.7.2.3 Sample Analysis

Data analysis was also performed using ChemStation for LC 3D Systems software, version Rev B.03.02[341]. Standard curves were generated by measuring the area under the curve for known concentrations of ADMA, SDMA and L-arginine and calculating ratios relative to the area under the curve for the internal standard (L-NMMA). ADMA, SDMA and L-arginine concentrations in the studied samples were calculated from "unknown sample/internal standard" ratios and using the standard curve to determine ADMA, SDMA and L-arginine concentrations.

2.8 Assessment of platelet response to NO

2.8.1 Whole blood impedance aggregometry

Whole blood impedance aggregometry is a technique to measure platelet aggregation *in vitro* (Cardinal & Flower 1980). After collection, blood samples were kept on the bench at room temperature for 20 minutes for stabilization, prior to aggregation studies. An electrode probe consisting of two metal wires was immersed in the blood sample, and small voltage was applied to the circuit. When platelets are exposed to an agonist (e.g. adenosine diphosphate (ADP)), they aggregate on the surface of the electrode. The accumulation of platelets adds electrical impedance to the circuit, which is proportional to the extent of platelet aggregation and is quantified by the aggregometer, in Ohms (Ω). The electrical impedance between two electrodes in whole blood samples is recorded over time.

2.8.2 Platelet response to adenosine diphosphate in whole blood

Platelet aggregation in whole blood was studied using a dual-channel impedance aggregometer (Figure 2-11 A, Model 560, Chrono-Log, Havertown, Pennsylvania, USA)

as previously described (Chirkov et al. 1999). In brief, tests were performed at 37 ºC and stirred using siliconized stir bars (Chrono-log Corporation, US) with stirring speed of 900 rpm. Samples of whole blood were diluted two-fold with normal saline with a final volume of 1 mL in polystyrene cuvettes (Chrono-log Corporation, US) and pre-warmed for 5 minutes at 37 ºC. Electrodes were then inserted into the samples and the aggregometer was calibrated for each sample relative to a resistance of 20Ω before the induction of aggregation by adding 2.5mmol/L ADP. Aggregation was evaluated as the net change in resistance (ohms Ω). All tests were run in duplicates.

A. B.

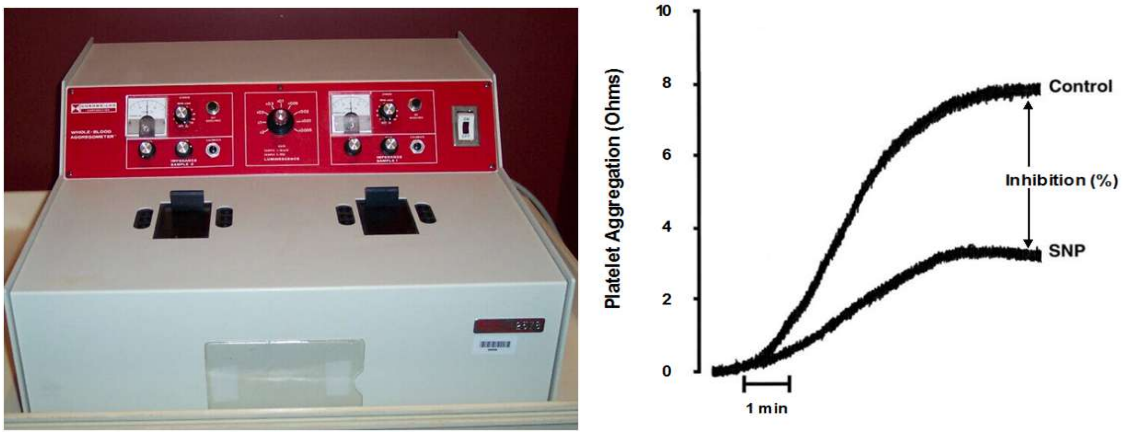

Figure 2-11: Assessment of platelet responsiveness to NO.

A. Platelet aggregation and platelet NO responsiveness was evaluated in whole blood samples via impedance aggregometry.

B. Inhibition of adenosine diphosphate-induced platelet aggregation in whole blood by sodium nitroprusside (SNP; 10µmol/L) as an NO donor. On the vertical axis is platelet aggregation expressed in Ohms and on the horizontal axis time in minutes.

2.8.3 Platelet response to SNP in whole blood

The NO donor SNP (10μmol/L) was utilized to quantitate platelet responsiveness to NO. SNP was added to samples 1 minute before ADP. Duration of pre-incubation with SNP was estimated as the optimal in previous experiments (Chirkov et al. 1999). Inhibition of aggregation was evaluated as a percentage comparing the extent of maximal aggregation in the presence and absence of SNP (Figure 2-11 B).

2.9 Measurement of total MPO release in neutrophils

MPO released from neutrophils was assayed with an MPO-ELISA kit (Mercodia Developing diagnostics, Uppsala, Sweden). Neutrophil cell suspension of $5x10^6$/mL was pre-incubated with BNP (1μmol/L) for 10 minutes. After stimulation with PMA for 20 minutes at room temperature, the cell suspension was immediately placed on ice to avoid further release of MPO and centrifuged at 550*g* for 10 minutes at 4°C, the supernatant was retained. Control assays were performed with un-stimulated cells in HBSS buffer with proper vehicle control. Supernatant was assayed immediately in triplicate according to manufacturer's instructions.

2.10 Determination of Plasma Thrombospondin-1

Plasma thrombospondin-1 levels were determined by ELISA according to manufacturer's instructions (Quantikine®, R&D Systems, and US). Briefly, blood was collected into heparinised tube and placed on ice immediately. Samples were centrifuged at 1800*g* for 15 minutes at 4°C. The supernatant was retained and centrifuged again at 10,000*g* for 10 minutes at 4°C to remove any residual platelets and red blood cells. The supernatant was collected and stored at -80°C until assayed. Samples were assayed in triplicate with

coefficients of variation determined from 5 replicate samples over 6 consecutive runs. Intra-assay CV was 6.6% and inter-assay CV was 6.3%.

2.11 Other parameters examined

(a) The NT-proBNP level was assayed within 2 hours after venesection. The determination of plasma NT-proBNP was performed at admission and follow up via Roche CARDIAC® proBNP (NT-proBNP assay) (Roche Diagnostics, Mannheim, Germany).

(b) CRP levels were determined with a high-sensitivity CRP assay (hs-CRP) (Beckman Immage Immunochemistry System, Fullerton, California, USA).

2.12 Chemicals

BNP was purchased from BACHEM (Bubendorf, Switzerland) and stock solution was prepared with deoxygenated water and aliquoted and stored at -80°C. cGMP radioimmunoassay kits was purchased from Biomedical Technologies Inc. (Stoughton, MA, USA). MPO-ELISA kit (Mercodia Developing diagnostics, Uppsala, Sweden). CM-H was purchased from Enzo Life Sciences (San Diego, CA, USA). Stock solutions of CM-H (400mmol/L) were prepared in dimethyl sulfoxide (DMSO) and kept at -20℃; Working solutions of CM-H (2mmol/L for neutrophil O_2^- and 400μmol/L for whole blood ROS) were prepared daily in Krebs - Hepes buffer (pH 7.4) containing 5.786g/L NaCl, 0.35g/L KCl, 0.368g/L $CaCl_2$, 0.296g/L $MgSO_4$, 2.1g/L $NaHCO_3$, 0.142g/L K_2HPO_4 , 5.206g/L Na-Hepes, 2g/L D-glucose, pH 7.4, in the presence of 25μmol/L deferoxamine and 5μmol/L diethyldithiocarbamate. 8-pCPT-cGMP was purchased from Biolog life science institute (Bremen, Germany). PMA, fMLP, and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Chapter 3: Evaluation of BNP-triggered biochemical signaling in neutrophils: physiology and pathology

3.1 Background

Plasma levels of B-type natriuretic peptide (BNP) increase dramatically in congestive heart failure (HF) (Gardner et al. 2003; Hobbs, FD et al. 2002). BNP is one of the most important biomarkers for the diagnosis, risk stratification, and prediction of death in patients with congestive HF (Braunwald 2008; Lainchbury, Espiner, Frampton, et al. 1997; Lainchbury, Espiner, Nicholls, et al. 1997; Palazzuoli et al. 2012). Natriuretic peptides (NPs) are generally regarded as cardioprotective hormones for their ability to reduce blood pressure, plasma volume, and myocardial infarct size (Burley & Baxter 2007; Molkentin 2003). However, other investigators reported increased infarct size after ANP administration in a murine model of myocardial infarction (MI) (Houng et al. 2009). Moreover, although NPs are thought to limit the progression of HF (Munagala, VK, Burnett, JC & Redfield, MM 2004), the administration of recombinant BNP (Nesiritide) was found to be ineffective in acute HF patients (O'Connor et al. 2011). Thus, although NPs have been viewed as a "compensatory" neurohormonal system in HF, it is important to delineate the role of BNP and its potential variability in HF patients on a biochemical basis.

The beneficial effects of NPs are attributed to the formation of the secondary messenger cGMP, which has smooth muscle relaxing and vasodilating effects (Daniels & Maisel 2007; Murad 2006). cGMP acts as an important mediator of many signaling events by interacting with PKGs, CGN, and cGMP-regulated cyclic nucleotide phosphodiesterases (Figure 1-10) (Lincoln & Cornwell 1993), which means that through these receptor proteins in the cell,

cGMP could theoretically regulate a number of intracellular processes, exerting negative metabolic and functional effects in myocardium and other functional sites (Murad 1994; Shah et al. 1994).

Intracellular cGMP formation is dependent on two forms of guanylyl cyclases: soluble guanylyl cyclase (sGC) and particulate guanylyl cyclase (pGC) (Lucas et al. 2000). As described in section 0, BNP acts through binding to natriuretic peptide receptor-A (NPR-A) linked pGC, which results in the generation of cGMP (Garbers 1992). Therefore, to study BNP-triggered biochemical signaling, the primary target should be stimulation of formation of cGMP. Increased cGMP formation has previously been demonstrated with BNP administration in plasma, in tissue/cells culture (Bryan et al. 2007; Forfia et al. 2007; Huntley et al. 2006; Jaiswal 1992; Piggott et al. 2006), as well as in isolated cells such as neutrophils (Matsumura et al. 1996). Furthermore, nitric oxide (NO)-induced cGMP formation through activation of the cytosolic sGC has been studied extensively, as mentioned in section 1.2.5.1.2.2. It has also been reported that both NO and NO donors such as SNP, increased cGMP production in human neutrophils (Elferink & de Koster 1992; Morikawa et al. 1995).

The effects of NPs and NO on superoxide (O_2^-) release from cells are less clear-cut. Two groups have reported that A-type natriuretic peptide (ANP) (Wiedermann et al. 1992) and BNP (Garlichs et al. 1999) sensitized mechanisms for O_2^- production in human neutrophils. However, Lin et al. reported that BNP inhibited angiotensin II (AngII)-induced O_2^- release in cultured rat cardiomyocytes (Lin et al. 2012). As regards NO, data are more consistent: NO has been reported to suppress O_2^- release both in neutrophils (Clancy, Leszczynska-Piziak & Abramson 1992) (Moilanen et al. 1993) and in cardiomyocytes, and it has been

shown that these effects are also seen with cGMP. Therefore, it is potentially important to determine whether BNP might differ from NO, and if so, how.

The impetus for such investigations has been increased by recent report that under some circumstances, both BNP and cGMP may increase catecholamine release (Chan et al. 2012), in which case these might be "paradoxical" effects *in vivo*, but presumably not *in vitro*.

3.2 Can we quantitate cGMP generation by BNP stimulation in neutrophils?

3.2.1 Methods:

3.2.1.1 Subject selection

A total of 19 healthy subjects, aged from 23-55, with no previously recorded cardiac diseases/dysfunction and 5 acute HF patients (selection based on section 2.1) were recruited in this study. The protocol was approved by the institutional Ethics of Human Research Committee, and informed consent was obtained prior to study entry.

3.2.1.2 Neutrophil isolation

Refer to section 2.3

3.2.1.3 Intracellular cGMP determination

Refer to section 2.4.

3.2.2 Results:

In neither control subjects (n=19) nor acute HF patients (n=5) did BNP significantly increase neutrophil cGMP production. For example, in control subjects the increase in cGMP production with maximal BNP concentration (1μmol/L) was 8±6% (p=NS) while SNP increased neutrophil cGMP production by 17±5% (p<0.05) (Figure 3-1).

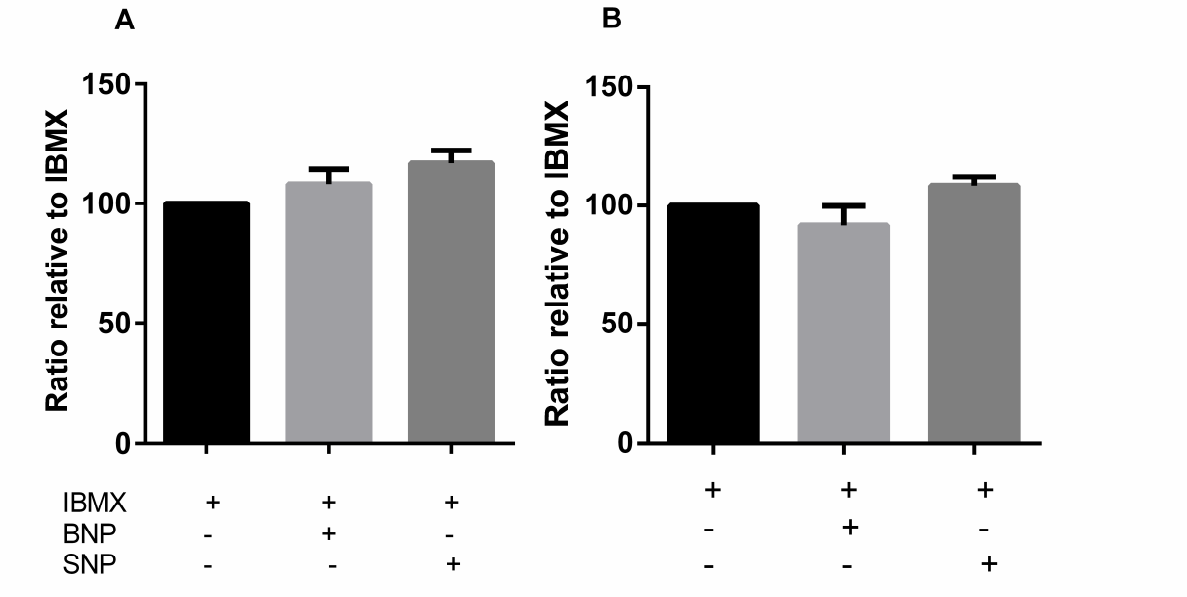

Figure 3-1: Effect of BNP and SNP on cGMP levels in human neutrophils.

(A): Control subjects (n=19); (B): Acute HF patients (n=5). Neutrophils were preincubated with or without BNP (10minutes) or SNP (1minute) in the presence of IBMX. Results were expressed as ratio relative to IBMX alone (Mean±SEM).

3.2.3 Discussion

In this study, we were unable to quantitate the incremental cGMP production in neutrophils treated with BNP despite the previously documented activation of pGC by

BNP (Matsumura et al. 1996). However, Weidermann et al (Wiedermann et al. 1992) also were unable to detect increases in cGMP content in neutrophils following ANP exposure as were Su et al. with CNP in myocardium (Su, Scholz & Weiss 2005). This could be due to the technical limitations of the assay, which does not allow for the localized detection of cGMP generation in proximity to membrane-bound pGC, but instead operates with the total cell volume (Piggott et al. 2006; Su, Scholz & Weiss 2005). The existence of particulate and cytosolic sources of guanylyl cyclases may result in the distinct rather than uniform distribution of cGMP within cells. Furthermore, diffusion of the newly formed cGMP will likely be limited by the localization of phosphodiesterases (some of the phosphodiesterases and their isomers are soluble, while others are bound to plasma and intracellular membrane) which are able to hydrolyze cGMP (Francis, SH, Turko & Corbin 2001). The different subcellular sources of cGMP and the presence of cGMP phosphodiesterases represent potential explanations for a diversely localized elevation of cGMP within the cell (Piggott et al. 2006; Su, Scholz & Weiss 2005).

3.3 BNP effects on neutrophil O_2^- production in healthy control subjects

3.3.1 Introduction:

Neutrophil activation and infiltration is critical in inflammatory responses associated with various forms of heart disease, including myocarditis, acute myocardial infarction (MI) and atrial fibrillation (Friedrichs et al. 2014; Goldmann et al. 2009). The pro-inflammatory actions of neutrophils relate largely to NAD(P)H oxidase, which generates superoxide anion radical (O_2^-), and myeloperoxidase (MPO), which produces hypochlorous acid (HClO), upon neutrophil degranulation, a process known as the "neutrophil burst" (Weiss 1989).

It has become increasingly clear in recent years that the neutrophil burst is subject to physiological and pharmacological modification. Nitric oxide (NO) may act to suppress NADPH oxidase activation (Clancy, Leszczynska-Piziak & Abramson 1992; Moilanen et al. 1993), and appears to do so via stimulation of its major biochemical "receptor" sGC, with subsequent increase in cGMP concentration. Another cGMP generator is membrane-bound particulate guanylyl cyclase (pGC), which can be activated by B-type natriuretic peptide (BNP) via natriuretic peptide receptor A (NPR-A). BNP is released largely from atrial and ventricular myocardium in response to distension (Cowie & Mendez 2002) and/or inflammatory activation (Ogawa, T & de Bold 2012) and has been shown to exert vasodilatation and natriuretic effects. BNP has been suggested as a physiologically stabilizing hormone, for example in heart failure (HF) (Kellett 2006). Given that the main biochemical product of BNP release is cGMP, we hypothesized that BNP might, like NO, suppress the neutrophil burst. We therefore evaluated this putative effect in isolated neutrophils obtained from normal subjects. Given that protein kinase G (PKG) is activated

by cGMP, thus initiating a variety of intracellular effects (sequestration of cytosolic Ca^{2+}, suppression of extracellular Ca^{2+} influx, inhibition of phospholipases C and A2, etc.), this enzyme is an important regulator of neutrophil function (Werner et al. 2005; Wyatt, Lincoln & Pryzwansky 1993). In order to examine this final step within the BNP-induced cGMP signaling, we investigated the effect of a PKG inhibitor (KT5823) on the hypothesized suppression of the neutrophil burst by BNP.

3.3.2 Methods:

3.3.2.1 Subject selection

A total of 20 healthy subjects, aged from 23-55 years, with no previously recorded cardiac diseases/dysfunction were recruited in this study. The protocol was approved by the institutional Ethics of Human Research Committee (The Queen Elizabeth Hospital, Adelaide, Australia), and informed consent was obtained prior to study entry.

3.3.2.2 Neutrophil isolation

Refer to section 2.2.

3.3.2.3 Determination of neutrophil O_2^- release and MPO release

Refer to sections 2.5.3 and 2.9.

a) BNP effects on O_2^- release in nuetrophils

Neutrophil cell suspensions $1.7x10^6$ /mL were incubated with BNP (1μmol/L, 100nmol/L, 10nmol/L, and 1nmol/L) for 10minutes then stimulated with either the PKC agonist phorbol 12-myristate 13-acetate (PMA, 100nmol/L, incubated for 20 minutes) (Liu, FC et

al. 2012) or *N*-formyl-methionyl-leucyl-phenylalanine (fMLP, 1μmol/L, no incubation) before the addition of CM-H (0.2mmol/L).

b) BNP effects on MPO release in neutrophils

Neutrophil cell suspension of $5x10^6$ /mL was preincubated with BNP (1μmol/L) for 10minutes. After stimulation with PMA for 20minutes at room temperature, the cell suspension was immediately placed on ice to avoid further release of MPO and centrifuged at 550g for 10 minutes at 4°C. Control experiments were performed with unstimulated cells in HBSS buffer alone. Supernatant was collected and assayed immediately using the MPO-ELISA kit (Mercodia Developing Diagnostics, Uppsala, Sweden) according to manufacturer's instructions.

c) Comparisons with cell-permeable cGMP analogue

Neutrophil suspensions (1.7×10^6 cells/mL) were incubated with the cell permeable cGMP analogue 8-pCPT-cGMP (0.5mmol/L) for 10minutes and then stimulated with either PMA (100nmol/L), or fMLP (1μmol/L) before the addition of CM-H (0.2mmol/L).

d) Effect of protein kinase G inhibition

Neutrophil suspensions (1.7×10^6 cells/mL) were incubated with BNP (1μmol/L) followed by KT5823 (PKG inhibitor, 1μmol/L) or DMSO (vehicle control) for 10minutes, then stimulated with either PMA or fMLP before the addition of CM-H (0.2mmol/L).

3.3.2.4 Chemicals

Refer to section 2.12.

3.3.2.5 Statistical analysis

All normally distributed data are expressed as means ± SEM. Statistical significance was determined by Student's paired *t*-test. Effects of interventions were assessed by ANOVA. GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA, USA) was used. Values of $P<0.05$ were considered statistically significant.

3.3.3 Results

3.3.3.1 O_2^- and MPO generation during neutrophil burst

The rate of O_2^- accumulation significantly increased upon stimulation of neutrophils with PMA (approximately 13 fold increase), and fMLP (approximately 2.5 fold increase) (Figure 3-2). Specificity of O_2^- anion detection by the spin probe-CMH was confirmed by inhibition of EPR signal with the NAD(P)H oxidase inhibitor diphenyleneiodonium (DPI; see Methods). There was also a 2-fold increase of MPO release from PMA-stimulated neutrophils compared to baseline state (Figure 3-3).

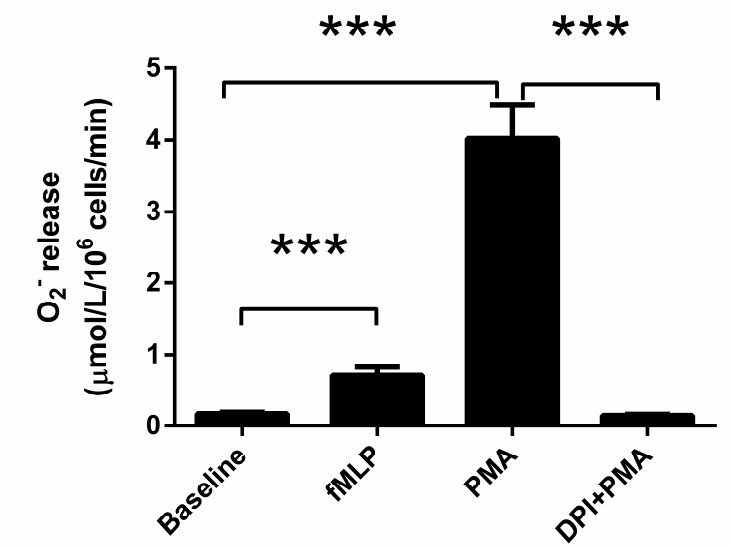

Figure 3-2: O_2^- production in neutrophils with both PMA (n=20) and fMLP (n=16) stimulation.

*Note suppression of PMA-induced O_2^- release with DPI. ***P<0.001*

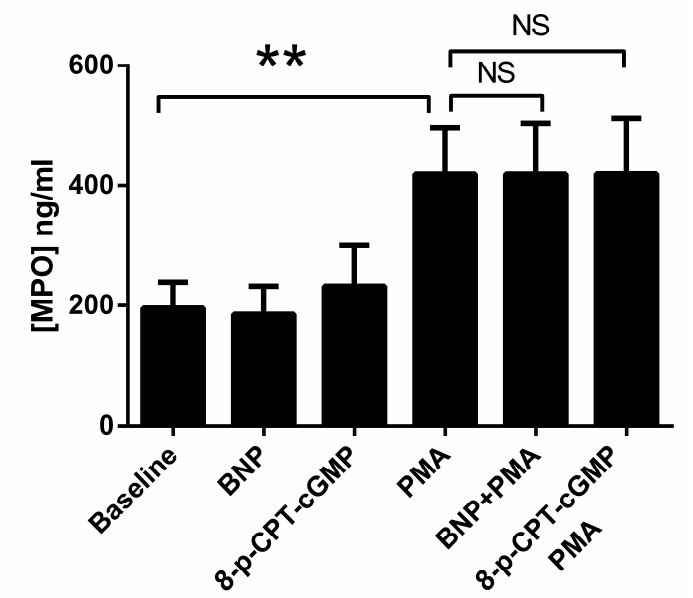

Figure 3-3: BNP and cGMP analog, 8-pCPT-cGMP effects on neutrophil MPO release.

*n=6; **P<0.01*

3.3.3.2 Effects of BNP and of cGMP analogue on neutrophil O_2^- and MPO generation

BNP caused concentration-dependent suppression of PMA-stimulated O_2^- release ($P<0.01$, ANOVA; Figure 3-4), with the maximum effect occurring at 1μmol/L. We therefore evaluated BNP effects at this concentration throughout the remainder of the study. BNP suppressed PMA-induced O_2^- release by 23±6% ($P<0.001$), and fMLP-induced O_2^- release by 24±8% ($P<0.05$; Figure 3-5). This effect of BNP is not affected by age or gender. Importantly, BNP in this concentration did not affect either basal O_2^- release from neutrophils (basal: 1.36±0.2 μM/10^6cells/minute, vs BNP: 1.31±0.2 μM/10^6cells/minute; n=13) or resting neutrophil MPO content or PMA-stimulated MPO release (Figure 3-3). Analogously, the cGMP analogue 8-pCPT-cGMP suppressed both PMA- and fMLP-induced O_2^- release (by 16% and 28% respectively; $P<0.05$, Figure 3-6), but had no statistically significant effects on resting neutrophil MPO content or PMA-stimulated MPO release (Figure 3-3).

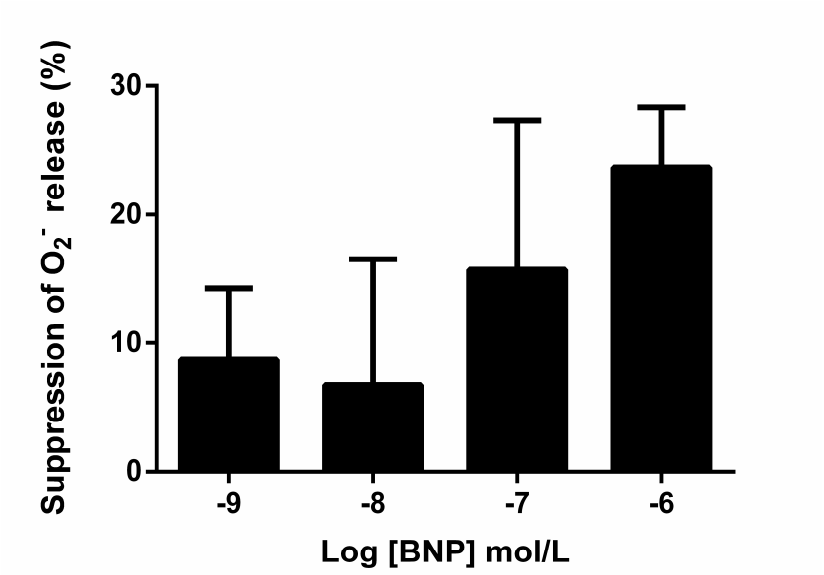

Figure 3-4: Concentration response relationship for BNP suppression of PMA-stimulated O_2^- release.

(n=20 for BNP concentrations of 1μmol/L and 1nmol/L, n=11 for BNP 100nmol/L, and n=12 for BNP 10nmol/L; P<0.01 ANOVA).

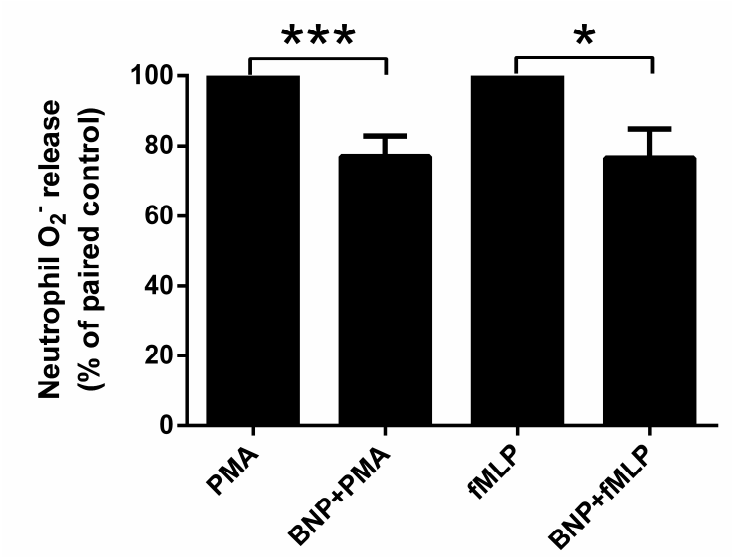

Figure 3-5: O_2^--suppressing actions of BNP.

*BNP (1μM) suppresses O_2^- release by both PMA (n=20) and fMLP (n=16) * P<0.05; *** P<0.001).*

3.3.3.3 Effects of PKG inhibition on neutrophil O_2^- generation

Given that O_2^- release was inhibited by 8-p-CPT-cGMP, but BNP did not cause a significant increase in intracellular cGMP content, we sought additional clarification for the signal transduction pathway for BNP-induced suppression of O_2^- release, utilizing the protein kinase G inhibitor KT5823 (1μmol/L). KT5823 alone exert no significant effect on PMA-induced (Δ -10±5%) or fMLP-induced (Δ -16±11%) O_2^- release. However, KT5823 attenuated by approximately 65% the effects of BNP on O_2^- release in the presence of

PMA ($P<0.05$, Figure 3-7), with a trend towards similar effect on fMLP-mediated O_2^- release.

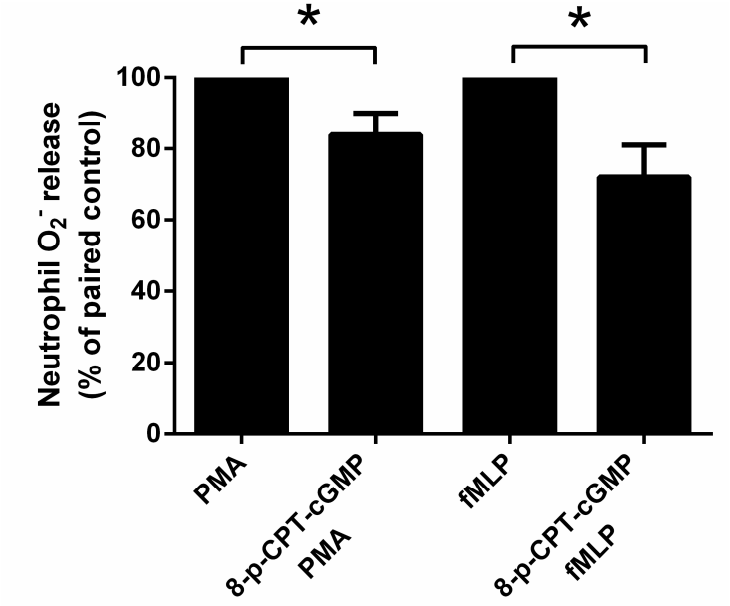

Figure 3-6: O_2^--suppressing actions of cGMP analogue.

8-pCPT-cGMP (0.5mmol/L) suppressed both PMA- (n=9) and fMLP- (n=8) induced neutrophil O_2^- generation.

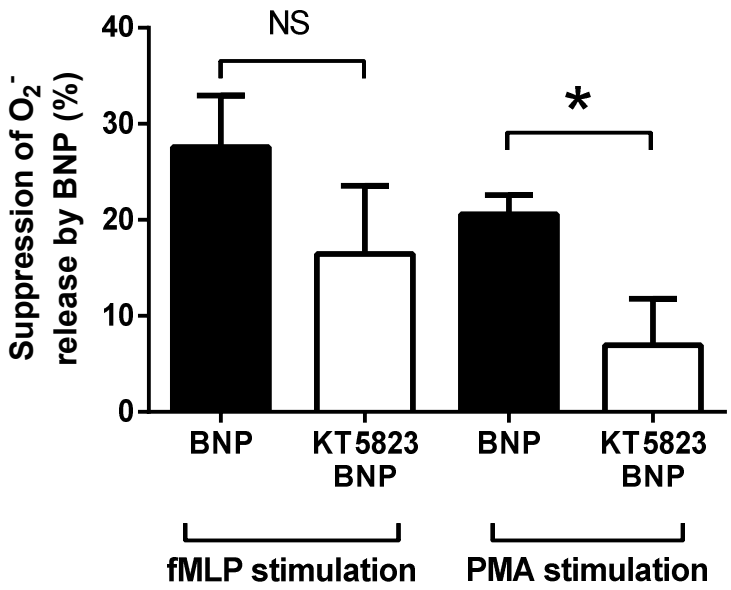

Figure 3-7: Effects of PKG inhibitor KT5823 (1μmol/L) on O_2^--suppressing actions of BNP.

*n=5; *P<0.05.*

3.3.4 Discussion

This study, performed in healthy subjects, has some important new findings regarding neutrophil physiology. Firstly, BNP inhibited neutrophil O_2^- generation stimulated by both PMA and fMLP. Secondly, this BNP effect was mimicked by a cGMP analogue, 8-pCPT-cGMP, and abolished by a PKG inhibitor KT5823. We therefore infer that this effect of BNP is likely to be mediated by the cGMP-PKG pathway.

Although the molecular mechanisms involved in BNP suppression of the neutrophil burst are not fully defined, the fact that suppression could be elicited with a variety of factors

suggested some possible mechanisms. The suppressing effect of BNP on neutrophil O_2^- generation stimulated by both PMA and fMLP is analogous to that previously reported in a study utilizing NO donors (4-aryl-substituted oxatriazol derivatives) (Moilanen et al. 1993). Similarly, previous studies showed that a cell-permeable cGMP analogue, N2,2'-O-dibutyryl guanosine 3':5'-cyclic monophosphate (db-cGMP) inhibited O_2^- generation stimulated by fMLP and platelet-activating factor. Also, db-cGMP inhibited fMLP-induced neutrophil degranulation (Ervens, Schultz & Seifert 1991; Wenzel-Seifert, Ervens & Seifert 1991).

The potential limitation of the current study is the uncertainty as to whether the currently demonstrated effects of BNP *in vitro* are physiologically relevant. While the utilized concentrations of BNP (Figure 3-4) may occur in the settings of increased BNP release with HF (Maisel, A 2002), we chose primarily 1μmol/L BNP throughout the study on the basis that this would provide reproducible and substantial response. Identification of precise threshold concentration for BNP effect *in vitro* would serve little purpose, given that it would not necessarily correspond to *in vivo* responsiveness: it is commonly the case for responsiveness to agonists *in vitro* to be lower than that seen *in vivo*.

Importantly, the cell-permeable cGMP analogue, 8-p-CPT-cGMP mimicked the effect of BNP (Figure 3-6). We also investigated the effect of PKG inhibition on the suppression of the neutrophil burst by BNP (Figure 3-7). KT5823 inhibited the effect of BNP and confirmed our postulate that this effect of BNP is cGMP-mediated.

The inhibition of neutrophil activation by BNP is of potential importance not only in host defense circumstances, but also in cardiovascular diseases, such as acute MI, AF, and HF, in which neutrophil-mediated inflammatory response plays a major role. Wiedermann and colleagues demonstrated that ANP inhibited fMLP-induced chemotaxis of neutrophils

(Wiedermann et al. 1992); and that stimulation of migration of neutrophils by fMLP is inhibited in the presence of ANP. In isolated rat cardiomyocytes, BNP also limits O_2^- generation (Lin et al. 2012); these myocardial effects also appear to be modulated by cGMP-dependent signaling (Laskowski et al. 2006).

In a cardiovascular context, we speculate that BNP may function to limit inflammatory responses in the presence of tissue infiltration by neutrophils. The current data raise the important issue of whether this may be relevant to limitation of myocardial injury following onset of infarction.

3.4 BNP effects on neutrophil O_2^- production-acute and chronic HF

3.4.1 Introduction:

B-type natriuretic peptide (BNP) has a wide range of important physiological effects, including natriuresis, diuresis, vasodilation, and inhibition of rennin-angiotensin-aldosterone system RAAS and sympathetic nervous systems (Kita et al. 1989; Lang et al. 1991; Wambach & Koch 1995). BNP is involved in regulation of cardiac and renal homeostasis, with beneficial effects mediated by stimulation of cellular membrane-bound particulate guanylyl cyclase (pGC), leading to formation of cGMP, and resulting in smooth muscle relaxing and vasodilating effects (see (Zois et al. 2014) for review).

Congestive heart failure (HF), which remains a major health-care problem (Go et al. 2013; Nieminen et al. 2006), is associated with BNP up-regulation (Gardner et al. 2003; Hobbs, FD et al. 2002). In theory, this should tend to restore cardiovascular homeostasis. Early clinical trials showed that systemic infusion of Nesiritide, a recombinant human BNP, improves hemodynamic parameters in acutely decompensated hearts (Abraham et al. 1998; Chandra et al. 2008; Colucci et al. 2000; Hobbs, RE et al. 1996; Mills et al. 1999). However, the ASCEND-HF trial (O'Connor et al. 2011) demonstrated that Nesiritide, relative to placebo, did not reduce the rate of death and re-hospitalization after 30 days' therapy in patients with acute decompensated HF. Moreover, Nesiritide did not significantly reduce dyspnea when utilized in combination with standard HF therapies. This raises the possibility of attenuated BNP response in such patients. However, to date, the issue of variability in tissue responsiveness to BNP has received relatively little attention. While data in animal models of congestive HF provided some support for the

concept of "BNP resistance" (Baerts et al. 2012), this possibility has yet to be evaluated in humans.

It is increasingly clear that in many patients HF is associated with inflammatory activation within the myocardium, largely mediated by neutrophil infiltration and associated generation of reactive oxygen species (ROS), especially superoxide (O_2^-) (Amir et al. 2009; Charniot et al. 2008; Koba, Gao & Sinoway 2009; van Empel et al. 2006). There is evidence that subsets of leukocytes markedly and differentially modulate inflammatory activation (Vaduganathan et al. 2013). BNP is known to exert anti-inflammatory and anti-fibrotic effects. It may well be that suppression of O_2^- release by BNP (Liu, S et al. 2014) limits myocardial inflammation in many forms of acute HF (Neil, C et al. 2012; Ogawa, T et al. 2008) .

We have recently demonstrated in neutrophils obtained from healthy subjects that BNP suppresses phorbol 12-myristate 13-acetate (PMA)- and *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated O_2^- generation (Liu, S et al. 2014). The cell-permeable cGMP analogue (8-pCPT-cGMP) suppressed both PMA- and fMLP-induced neutrophil O_2^- release. Furthermore, an inhibitor of cGMP-protein kinase (KT5823) attenuated the suppressing effects of BNP on both fMLP- and PMA-associated O_2^- production. These findings therefore suggest that the O_2^- suppressing effect of BNP utilizes the pGC/cGMP signaling pathway.

However, to date the impact of incremental BNP release from the myocardium on neutrophil ROS release has not been evaluated in the context of HF, either acute or chronic. In the current study, in neutrophils obtained from patients with acute and chronic HF, we focused on the ability of BNP to suppress O_2^- generation by NAD(P)H oxidase, and (1) examined whether it is different from that in healthy subjects, (2) evaluated potential

mechanism(s) underlying this putative change and (3) determined whether such effects persist during chronic treatment for HF.

3.4.2 Methods:

3.4.2.1 Study Cohort:

A total of 29 healthy subjects with no previously recorded cardiac dysfunction and 45 HF patients admitted to a tertiary care hospital (The Queen Elizabeth Hospital, Adelaide, Australia) with a primary diagnosis of acute HF but without planned coronary revascularization and/or valve replacement were compared. Consistent with international guidelines (McMurray et al. 2012; Yancy et al. 2013), the diagnosis of acute HF was based on the presence of dyspnea at rest or on minimal exertion, together with physical and radiological signs of fluid overload and echocardiographic evidence of systolic and/or diastolic left ventricular (LV) dysfunction.

All patients underwent early assessment and simultaneous venesection after (median: 2 days) hospital admission for determination of acute responses to BNP. Following stabilization and at least 3 weeks' (median: 5 weeks) treatment, patients were approached regarding repeat evaluation (n=25). Plasma concentrations of NT-proBNP were determined at admission and follow up via Roche CARDIAC® proBNP (NT-proBNP assay) (Roche Diagnostics, Mannheim, Germany).

The study was approved by the Ethics of Human Research Committee of The Queen Elizabeth Hospital (Adelaide, Australia), and informed consent was obtained prior to study entry.

3.4.2.2 Blood sampling and preparation of neutrophils

Refer to section 2.2.

3.4.2.3 Electron Paramagnetic Resonance Spectroscopy measurement of ROS

Refer to section 2.5.

3.4.2.4 Immunoblot analysis of p47phox and phosphorylation of p47phox in neutrophils

Refer to section 2.6.

3.4.2.5 Assessment of Endothelial Function

Refer to section 2.7.

3.4.2.6 Assessment of Platelet Response to NO

Refer to section 2.8.

3.4.2.7 Chemicals

Refer to section 2.12.

3.4.2.8 Data analysis

All normally distributed data are expressed as means ± SEM or as median for skewed data. Statistical significance was determined by Student's paired *t*-test for normally distributed data and via paired Wilcoxon test for non-Gaussian data. Effects of interventions were assessed by ANOVA. We also sought to identify determinants of variable BNP effects and platelet NO response among HF patients by backwards stepwise multiple linear regression analysis. For BNP effects, left ventricular ejection fraction (LVEF), gender, diabetes

mellitus and therapy with aldosterone antagonists, ACE inhibitors and perhexiline were utilized as potential correlates. For NO responses, gender, NYHA class, diabetes mellitus and therapy with statins and aldosterone antagonist were considered as potential correlates. Clinical correlates of changes in neutrophil responses to BNP with treatment of HF were evaluated via univariate analysis followed by backwards stepwise multiple logistic regression: - parameters evaluated in this respect were patients' age, duration of therapy, change in NT-proBNP levels, change in NYHA functional status and hs-CRP levels. GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA, USA) and IBM SPSS software version 21 (Chicago, Illinois, USA) was used. Values of $P<0.05$ were considered statistically significant.

3.4.3 Results

3.4.3.1 Subject/patient characteristics and pharmacotherapy:

Table 3-1 summarizes the clinical characteristics of patients admitted with acute HF and control subjects. The two groups differed as regards symptomatic status, comorbidities (e.g. diabetes), and age. Moreover only the acute HF patients were receiving pharmacotherapy. At baseline, NT-proBNP levels were markedly elevated in acute HF patients. In approximately 80% of acute HF patients, there was a longstanding history of impaired LV function, with at least 1 previous admission: hence the background of extensive pharmacotherapy. Consistent with the known effects of treatment with ACE inhibitors and perhexiline on the cGMP-system (Chirkov & Horowitz 2007), platelet NO responsiveness was within the normal range (Table 3-1). Furthermore, plasma ADMA concentrations were significantly higher in both acute and chronic HF patients than those seen in control subjects.

Table 3-1: Patients/Control subjects characteristics and pharmacotherapy:

		Control (29)	Acute HF (45)	Treated HF (25)
Age (years±SEM)		44±3	71±2**	69±2
Gender (M/F) (%)		52/48	69/31*	63/37
Previous myocardial infarction (n/%)		0/0	6/13	4/16
Diabetes mellitus (n/%)		0/0	20/44**	13/52
LVEF %		-	35±2	34±3
NYHA class	I (n/%)	0/0	0/0	0/0
	II (n/%)	0/0	0/0	7/28$^{\#}$
	III (n/%)	0/0	24/53**	12/48
	IV (n/%)	0/0	21/47**	6/24
NT-proBNP (pg/mL; median)		<60	3613	2409
hs-CRP (mg/L; median)		1.0	14**	4.5$^{\#\#\#}$
Vascular NO responses (δAIx)	δ AIx Salbutamol	370±48	-	360±48
	δ AIx NTG	317±42	-	297±48
ADMA (μmol/L)		0.58±0.02	0.70±0.02**	0.68±0.02
SNP-induced inhibition of aggregation (%)		30±5	37±5	31±6
Therapy	ACE inhibitor (%)	0	49**	70$^{\#}$
	ARB (%)	0	11	7
	Statin (%)	0	51**	52
	Aldosterone antagonist (%)	0	33*	59$^{\#\#}$
	β-blocker (%)	0	60**	81$^{\#\#}$
	Perhexiline (%)	0	22*	15
	Digoxin (%)	0	29*	48$^{\#}$
	"Triple therapy" (%) †	0	18*	40$^{\#\#}$

- =not assessed. LVEF, left ventricular ejection fraction; NYHA class, New York Heart Association class; NT-proBNP, N-terminal pro B-type natriuretic peptide; hs-CRP, High-sensitivity C-reactive protein; AIx, augmentation index; ADMA, asymmetric

dimethylarginine; SNP, sodium nitroprusside; ACE, angiotensin-converting-enzyme; ARB, angiotensin receptor blocker; β-blocker, β-adrenoreceptor blocker.

*δAIx in response to salbutamol and NTG is expressed as area under the response-time curve. * P<0.05, ** P<0.0001, compared with control group. The comparison between acute HF and treated HF was based only on the 25 patients who had been evaluated at both time points ($^{\#}$ P<0.05, $^{\#\#}$ P<0.001, $^{\#\#\#}$P<0.0001).*

† "Triple therapy" refers to those patients receiving combinations of: (a) ACE inhibitor or ARB; (b) β-adrenoceptor antagonist; (c) Aldosterone antagonist.

3.4.3.2 Effects of BNP and cGMP analogue on whole blood ROS and neutrophil O_2^- generation in acute HF patients.

Baseline whole blood total ROS content did not vary significantly between control subjects and acute HF patients, nor did incubation with BNP for 10 minutes significantly alter ROS content in either control subjects or acute HF patients (two-way ANOVA, Figure 3-8 A). Furthermore, both BNP and the cell permeable cGMP analogue, 8-pCPT-cGMP, did not significantly affect O_2^- generation by neutrophils (without PMA or fMLP stimulation) in either normal subjects or acute HF patients (Figure 3-8 B). In control subjects, consistent with our previous observations (see section 3.3), BNP suppressed PMA- and fMLP-induced O_2^- generation by 23.6±4.7% and 33.3±5.8% respectively (P<0.05 for both). In contrast, there was significant attenuation of this effect of BNP in the acute HF patients relative to control subjects (Figure 3-9), with only 4.3±4.7% suppression of PMA-induced O_2^- generation by BNP (P=NS).

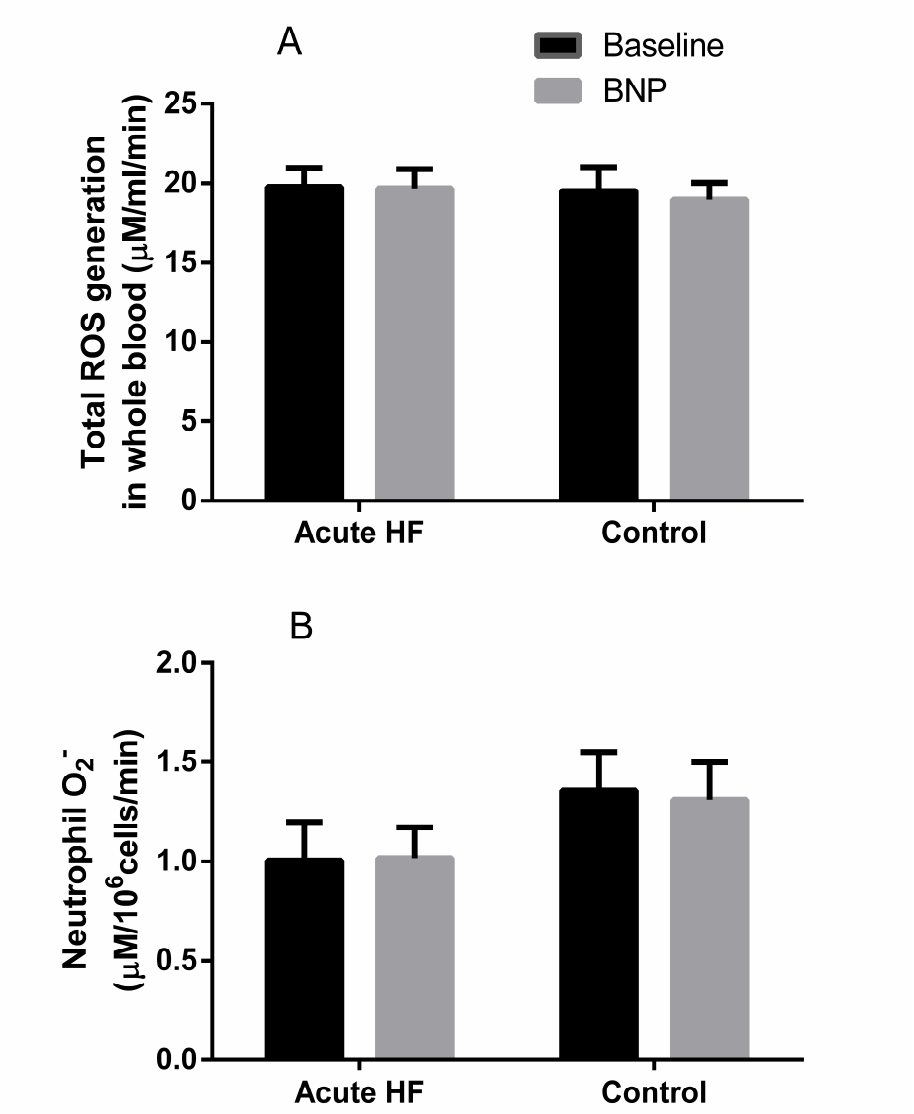

Figure 3-8: Lack of effect of BNP on whole blood ROS (A) and O_2^- generation by un-stimulated neutrophils (B).

Data for control subjects (n=14) and patients with acute HF (n=15) are compared, and no significant differences were observed. In no case did BNP have a significant effect in either control subjects or acute HF patients (two-way ANOVA). Neutrophil counts in whole blood were greater in HF patients than control subjects (on average: 137.5×103 vs 87.5×103 cells/sample).

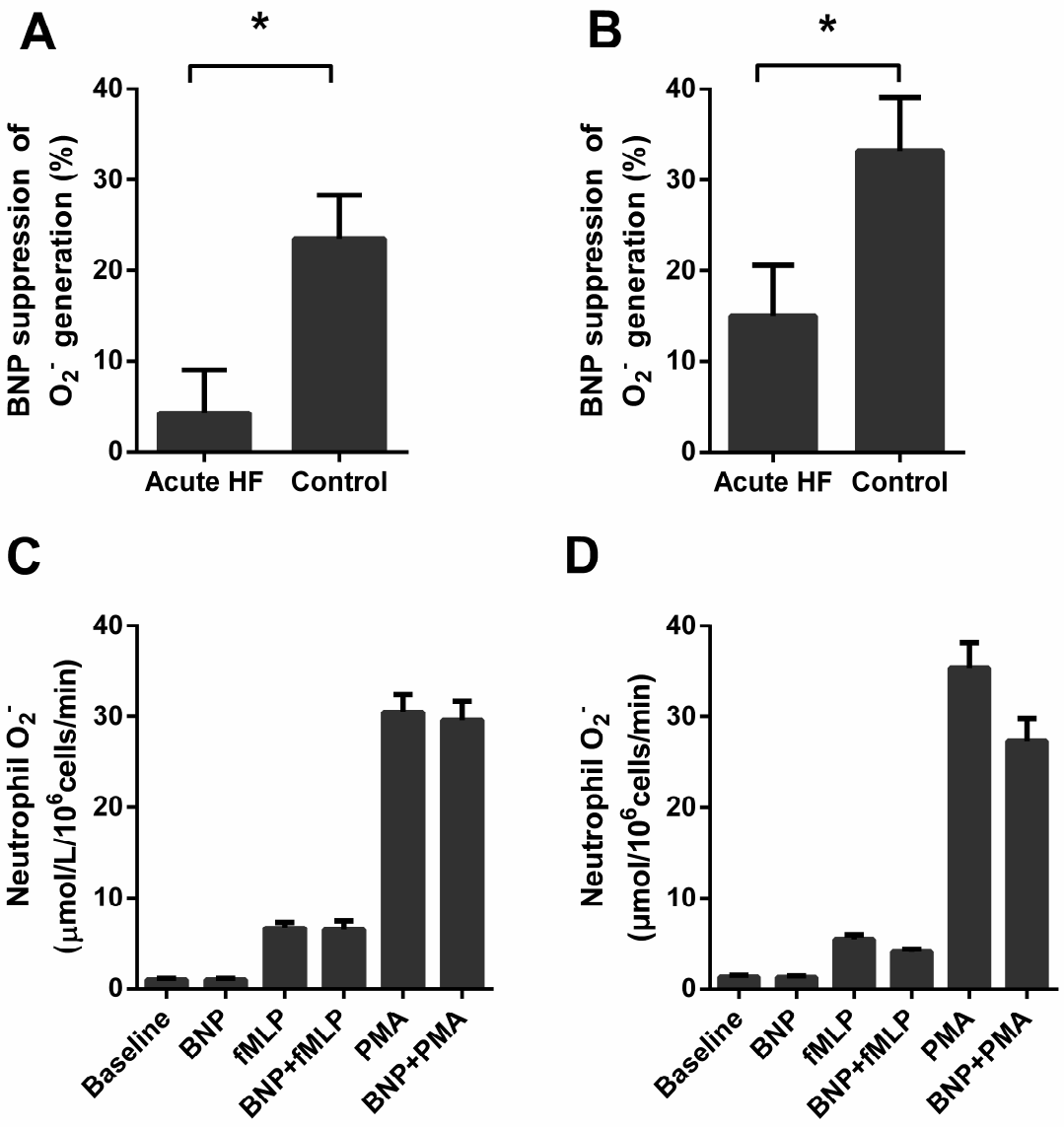

Figure 3-9: Comparison of BNP effects on PMA-stimulated (A) and fMLP-stimulated (B) O_2^- generation by neutrophils from acute HF patients (n=45) and control subjects (n=29).

*Raw data for acute HF patients (C) and control subjects (D). *P<0.05*

The observed decrease in BNP response in acute HF patients was not correlated with platelet response to SNP (Table 3-1). On multivariate analysis, no significant correlates of response to BNP could be identified. Furthermore, the effects of the cell permeable cGMP analogue, 8-p-CPT-cGMP, in suppressing PMA-induced O_2^- generation did not vary significantly between acute HF patients and control subjects (Figure 3-10).

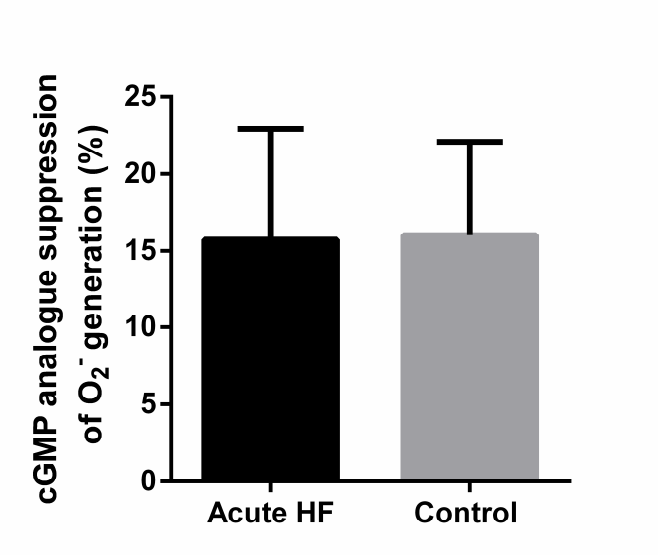

Figure 3-10: Suppression of O_2^- generation by the cGMP analogue 8-pCPT-cGMP (0.5mmol/L) in neutrophils from acute HF patients and control subjects.

3.4.3.3 BNP effects on phosphorylation of p47phox in acute HF patients

As p47phox phosphorylation is critical for NAD(P)H oxidase activation and priming, we compared the BNP effects on this process in neutrophils from healthy control subjects and patients with HF. In control subjects (n=7), fMLP-induced p47phox Ser345 phosphorylation was inhibited by 17.5±11% in the presence of BNP ($p<0.05$). In acute HF patients (n=9) no significant changes in p47phox phosphorylation occurred after incubation with BNP (P=0.25 versus control, Figure 3-11).

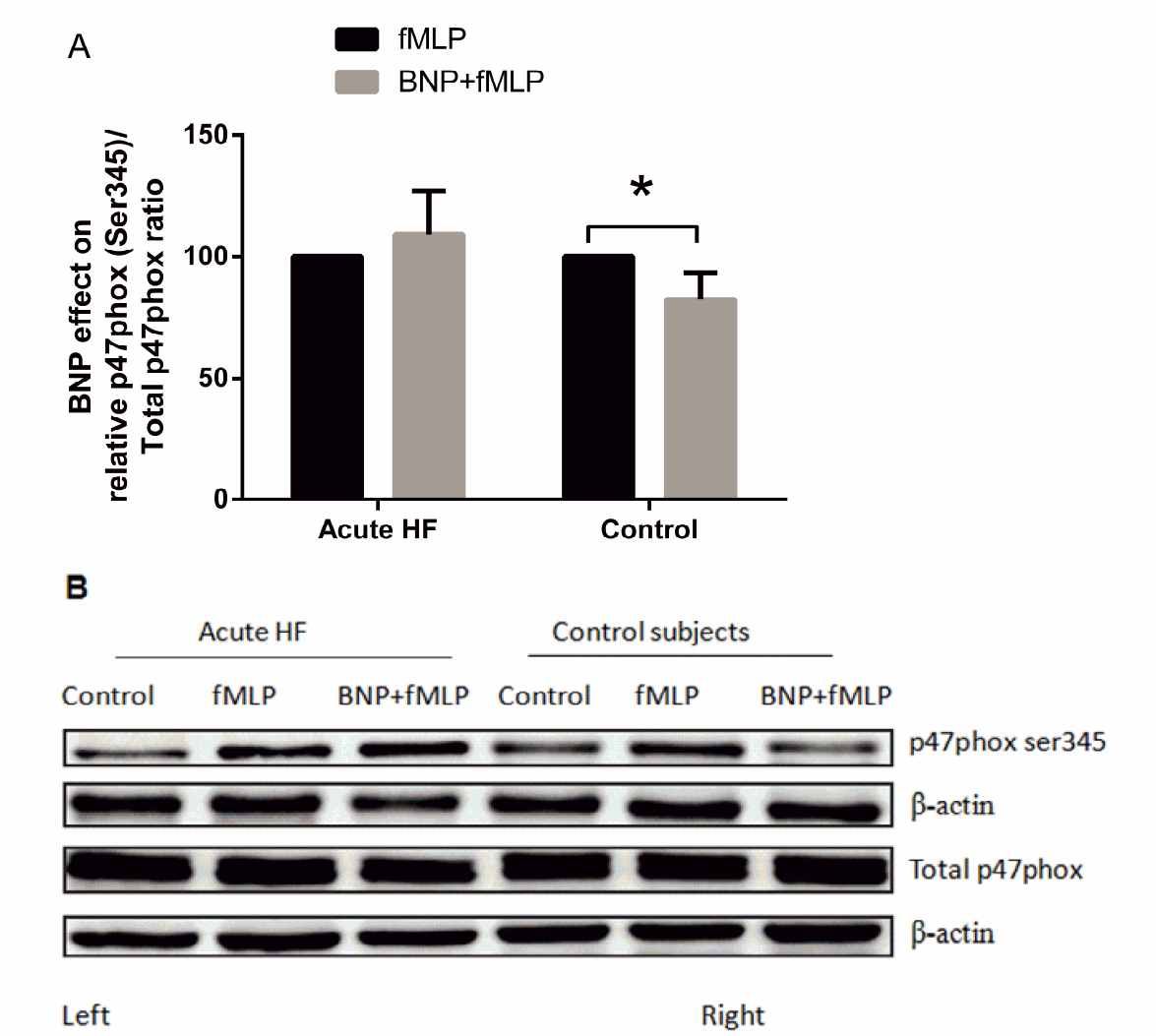

Figure 3-11: BNP effect on phosphorylation of p47phox Ser345.

A: In acute HF patients (n=9) there is loss of the BNP (1µmol/L) suppresses p47phox Ser345 phosphorylation stimulated by fMLP, which is seen in neutrophils from healthy subjects (n=7). *The relative ratio of p47phox Ser345/total p47phox for samples treated with fMLP was taken as control (100%). *P<0.05.*

B: Representative immunoblots: - acute HF patient and control subject. *Note suppression of phosphorylation of Ser345 by BNP in control subjects but not acute HF.*

3.4.3.4 Impact of chronic treatment of HF

Patients (n=25) receiving intensified treatment including ACE inhibitors, aldosterone antagonists and β-adrenoceptor antagonists were re-evaluated after 5 weeks (median) (Table 3-1). 52% of this group of patients had improved by at least 1 functional NYHA class. However, NT-proBNP plasma levels had not decreased significantly (median: 3613 to 2409 pg/mL). Paired evaluation of BNP responses before and after this incremental HF treatment (Figure 3-12) revealed partial restoration ($P<0.05$) of inhibition of O_2^- generation with treatment. On univariate analysis, decrease in NT-proBNP level was not predictive of this progressive re-sensitization (Figure 3-13). Moreover, on multivariate analysis no relationship was found between BNP effect on neutrophils and duration of treatment, age, NT-proBNP, hs-CRP, or NYHA class.

Neither *in vitro* platelet response to SNP, *in vivo* vascular responsiveness to salbutamol or NTG, or plasma ADMA concentrations correlated significantly with BNP effects on O_2^- generation.

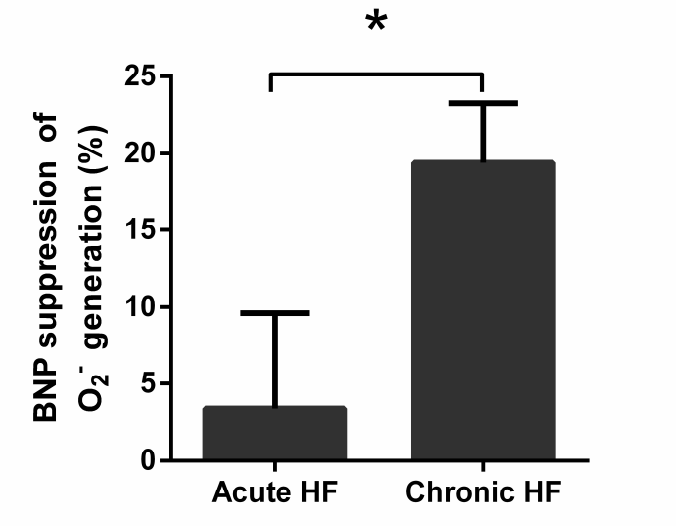

Figure 3-12: Impact of 3 weeks' therapy on BNP-induced suppression of PMA-stimulated O_2^- generation in neutrophils from HF patients.

*n=25; *P<0.05.*

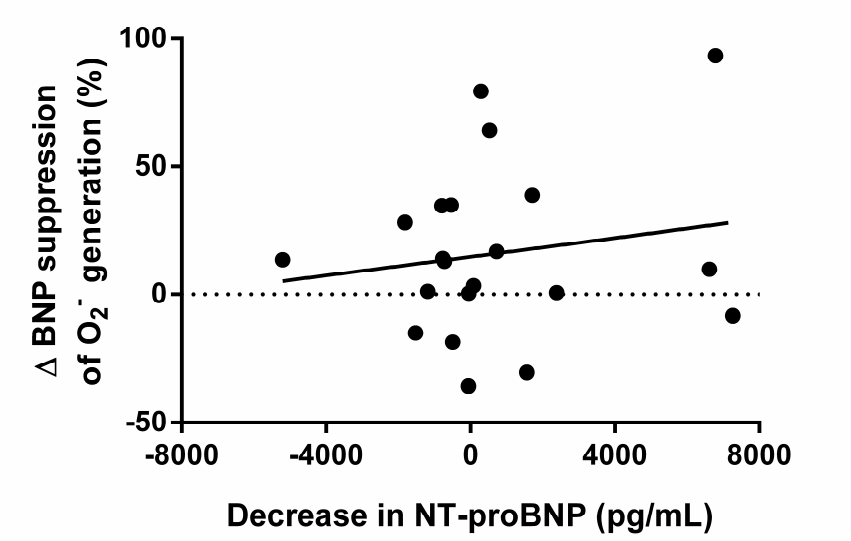

Figure 3-13: Correlation between fall in NT-proBNP level and re-sensitization of BNP effect after HF treatment.

r=0.17; P=0.47.

3.4.4 Discussion

We have characterized suppression of neutrophil O_2^- generation (PMA- or fMLP-stimulated neutrophil burst) by BNP as a measure of its physiological activity in the previous section (section 3.3). The central finding of the current study is that this effect of BNP is attenuated in acute HF patients, thus constituting "BNP resistance". This observation is of potential clinical importance for the management of acute and chronic HF, from both a mechanistic and therapeutic perspective. From a mechanistic point of view, HF is characterised by substantial release of BNP, largely due to ventricular distension (Yasue et al. 1994). Yet, this release fails to restore homeostasis. From a therapeutic standpoint, there is the issue of the potential benefit of treatment with synthetic BNP (Nesiritide) or other BNP analogues: to date such treatment has been disappointing (Abraham, Trupp & Jarjoura 2010), and there has been no consistent explanation in the literature for this treatment failure.

We have previously documented that suppression of O_2^- release in neutrophils in response to BNP is mediated by pGC/cGMP signalling (Liu, S et al. 2014). However, BNP did not significantly increase total neutrophil cGMP content, consistent with the relative paucity of natriuretic peptide receptor A (NPR-A) in neutrophils(Thom et al. 1997). Therefore, in the present study we did not evaluate cGMP accumulation in neutrophils in response to BNP because of lack of a reliable detection/quantitation method. Instead, we focused on the effects of BNP on NAD(P)H oxidase function.

The stimulation of neutrophils by PMA and fMLP, with the subsequent phosphorylation of p47phox, one of the cytosolic components of NAD(P)H oxidase, is the key process that controls and regulates the NAD(P)H oxidase activation (El-Benna et al. 2009; Liu, S et al. 2014). ROS released upon NAD(P)H oxidase activation can damage surrounding tissues.

In the current study, it was found that BNP administration leads to suppressed phosphorylation of p47phox in normal subjects, and that this effect tends to be diminished in acute HF. Theoretically, this change may represent a desensitization of NPR-A or impairment of pGC function in these patients, and may not be the only change in either NAD(P)H oxidase or neutrophil function induced in the presence of BNP.

Scientific evidence to date to substantiate a condition of tissue resistance to BNP in congestive HF has been limited. It has been reported that in chronic HF patients the vasodilator effects of BNP are impaired (Nakamura, M et al. 1998), and suggested that high endogenous BNP levels are associated with the down-regulation of cardiac BNP receptors, contributing to the progression of HF (Tsutamoto, Wada, Maeda, Hisanaga, Maeda, et al. 1997). Furthermore, in dogs with severe congestive HF induced by rapid ventricular pacing, both the vasodilator response to BNP and the associated cGMP generation were attenuated in comparison with controls (Matsumoto et al. 1999). Importantly, BNP-induced increases in plasma cGMP level were lower in HF dogs compared with control animals (Lainchbury et al. 2000).

Furthermore, NPR-A protein levels are reduced in kidney membranes of mice with HF induced by aortic constriction (Bryan et al. 2007), and expression of the cardiac NPR-A has been reported to be diminished in an animal model of HF (Dickey et al. 2007). Also, density of NPR-A in the heart and coronary arteries of humans with ischemic HF is decreased (Singh, G et al. 2006). Prolonged ANP exposure reduced NPR-A receptor density in a number of cell lines with consequent implications of diminution in responsiveness to both ANP and BNP (Flora & Potter 2010).

Although a direct assessment of BNP receptor in the current setting would be desirable, the low number of NPR-A receptors on neutrophils (Thom et al. 1997) would render the

detection of partial desensitization difficult. However, it is clear from the current results that distal to NPR-A(pGC) the BNP signalling pathway is intact. Indeed, while the suppressing effects of BNP on p47phox phosphorylation and O_2^- release are diminished in neutrophils from HF patients, responsiveness to the cell permeable cGMP analogue, 8-pCPT-cGMP, was preserved. This implies HF-associated dysfunction of the NPR-A(pGC), potentially due to both receptor desensitization and susceptibility of pGC to oxidative stress from O_2^-. Despite this role of O_2^-, there was no correlation between responsiveness to BNP and platelet/vascular responsiveness to NO donors in individual patients, suggesting that mechanisms of "BNP resistance" are different to those of "NO resistance", based on the impact of oxidative stress on soluble GC (Chirkov & Horowitz 2007).

With treatment of HF, half of the patient cohort improved symptomatically, although NT-proBNP levels generally remained elevated. This raises the possibility that more intense and/or prolonged HF therapy might have both reduced NT-proBNP levels and concomitantly significantly improved neutrophil responses to BNP. Indeed, there is some evidence that "BNP-guided therapy" of HF is associated with improved outcomes (Porapakkham et al. 2010): one possible mechanism underlying this would be limitation of neutrophil O_2^- release and thus amelioration of oxidative stress.

Given that these investigations have been performed in neutrophils rather than myocardial cells, it is appropriate to review briefly the roles of neutrophils as modulators of the natural history of HF. Previous investigations have documented elevation of neutrophil count (Arruda-Olson et al. 2009), and elevation of neutrophil-to-lymphocyte ratio (Benites-Zapata et al. 2015) in community studies of HF. Furthermore, both in experimental models of HF (Kawakami et al. 2004) and clinically (Ladich, Otsuka & Virmani 2014), neutrophil infiltration of myocardium is a common finding. Kawakami et al. (Kawakami et al. 2004)

documented that in BNP-transgenic mice, the number of neutrophils infiltrating areas of infarcted myocardium was significantly increased. Thus it might be postulated that chronic elevation of BNP levels (as in HF) results both in increased neutrophil presence in the myocardium and loss of the inhibitory role which BNP normally plays in modulating O_2^- release.

The question arises as to whether attenuated tissue responsiveness to BNP in acute and chronic HF is deleterious. Superficially, the current data add to this suspicion: increase in net O_2^- generation associated with the "neutrophil burst" would be likely to induce aggravation of redox stress, compounding the impact of diminution of hemodynamic and natriuretic effects of BNP. It has also recently emerged that BNP may increase catecholamine release (Chan et al. 2012). It remains to be determined whether this effect is also attenuated in HF: if so, the deleterious effects of "BNP resistance" might be mitigated.

As stated above, the current data can be taken as a theoretical support for "BNP-guided therapy" in HF. Furthermore, it may be possible to maintain tissue responsiveness to BNP via appropriate HF pharmacotherapy: in the recently published PARADIGM trial (McMurray et al. 2014), the combination of valsartan with the neprilysin inhibitor sacubitril (AHU377) was associated with markedly improved outcomes despite greater BNP accumulation in plasma.

Although natriuretic peptides are potentially cardioprotective, our results may help rationalize the findings of a recent large clinical trial (O'Connor et al. 2011) that treatment with recombinant BNP fails to protect HF patients from rehospitalization and death. Thus, amelioration of "BNP resistance" emerges as a relevant therapeutic target in acute HF.

3.5 Does increased BNP release "automatically" down-regulate neutrophil O_2^- generation: studies in Takotsubo Cardiomyopathy.

3.5.1 Introduction

Takotsubo Cardiomyopathy (TTC), also called stress-induced cardiomyopathy, apical ballooning syndrome or broken heart syndrome, is often confused with acute coronary syndrome (ACS) (Bybee et al. 2004; Gianni et al. 2006). TTC occurs predominantly in ageing women, and episodes of chest pain corresponding to the onset of TTC are usually associated with emotionally or physically stressful events. Irrespective of symptoms, the diagnosis of TTC tends to be made on the basis of transient segmental left ventricular (LV) wall motion abnormalities particularly involving the LV apex or mid-ventricle without obstructive coronary artery disease (CAD) (Gianni et al. 2006). Although the syndrome was first recognized and reported by a Japanese group (Dote et al. 1991) it is now recognized that TTC occurs frequently in Caucasian populations, and indeed that it accounts for up to 10% of "heart attacks" in women aged more than 50 years (Raman et al. 2014).

The pathophysiology of the TTC syndrome remains incompletely elucidated at present. Among pathophysiological mechanisms which have been proposed are multivessel coronary vasospasm, abnormalities in coronary microvascular function or spasm, catecholamine-mediated myocardial "stunning", coronary emboli with spontaneous fibrinolysis and/or transient obstruction of the LV outflow tract (Gianni et al. 2006; Prasad, Lerman & Rihal 2008). However, more recently, it has emerged that TTC is primarily an inflammatory "myocarditis", with cardiac magnetic resonance imaging (MRI)

investigations revealing extensive, although mainly apical, oedematous reactions (Neil, CJ et al. 2012).

The cause of inflammation in TTC is at present not well delineated. Excessive release of catecholamines has been suggested to induce redox stress via release of ROS and promote inflammatory processes, resulting in myocyte dysfunction and apoptosis. Apart from cardiac MRI scanning (Eitel et al. 2010), inflammation has been documented directly via endomyocardial biopsies (Nef et al. 2007; Yoshida et al. 2007). Experimentally, a British group has provided evidence that TTC may be triggered by β_2-adrenoceptor stimulation and Gi-based signaling, but did not delineate the basis for inflammatory activation (Paur et al. 2012).

A number of investigations have reported markedly elevated plasma levels of B-type natriuretic peptide (BNP) (Akashi et al. 2004; Grabowski et al. 2008) or NT-proBNP (Nef et al. 2008) despite the fact that the LV filling pressure is not generally elevated in TTC (Akashi et al. 2003; Park et al. 2009). The marked and persistent elevation of NT-proBNP/BNP levels in TTC is correlated with both the extent of catecholamine increase and the severity of LV systolic dysfunction (Nguyen et al. 2011). Furthermore, a study conducted by Morel et al. (Morel et al. 2009) demonstrated that in TTC patients, inflammatory activation corresponded to the extents of impairment of LV function and neurohormonal activation. For example, C-reactive protein (CRP) levels were correlated to left ventricular ejection fraction (LVEF) and BNP levels, and plasma leukocyte counts were correlated both to BNP and noradrenaline levels (Morel et al. 2009). *These data extend the argument that the predominant stimulus for BNP release in TTC is inflammatory rather than myocardial distension.*

We have shown (see section 3.3 and 3.4) that BNP inhibited O_2^- generation in stimulated neutrophils of healthy subjects, and this effect is attenuated in acute HF patients. Given the presence of dramatically increased plasma NT-proBNP and BNP levels in acute TTC patients as well, the question arises: - "Does increased BNP release "automatically" down-regulate neutrophil response?" *Therefore, the objective of the present study was to determine (1) whether BNP-induced suppression of neutrophil O_2^- release is also present during the acute stages of TTC; (2) what are the determinants of BNP effect in individual patients with TTC.*

3.5.2 Methods

3.5.2.1 Study cohort:

Patients with TTC were prospectively identified according to the Mayo Clinic criteria (Madhavan & Prasad 2010). The study was approved by the institutional Ethics of Human Research Committee of The Queen Elizabeth Hospital (Adelaide, Australia), and informed consent was obtained prior to study entry.

All acute TTC patients underwent routine clinical assessment, including ECG monitoring, transthoracic echocardiography and coronary angiography. Additionally, blood samples were taken for determination of NT-proBNP and hs-CRP levels, and wherever possible (based on clinical stability and absence of contra-indications) cardiac MRI was performed to (i) exclude MI and (ii) quantitate oedema via T2-based imaging (Neil, C et al. 2012). Plasma levels of metanephrine and normetanephrine were determined at the time of diagnosis. All acute TTC patients had blood taken for neutrophil evaluation 0.5-5.5 days (mean 2.5±0.3 days) post onset of symptoms. Patients were re-approached for evaluation of BNP response in neutrophils at least 3 months post onset of symptoms.

3.5.2.2 Blood sampling and preparation of neutrophils

See section 2.2

3.5.2.3 BNP effects on neutrophil O_2^- generation by EPR spectroscopy

Refer to section 2.5.3

3.5.2.4 Platelet aggregatory and determination of platelet response to SNP

Refer to section 2.8

3.5.2.5 Chemicals

Refer to section 2.12

3.5.2.6 Data analysis

All normally distributed data are expressed as means ± SEM. Statistical significance was determined by Student's t test for paired normally distributed data. GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA) was used. Values of $p<0.05$ were considered statistically significant.

Comparisons were made between TTC patients (n=19), and control subjects (n=16, aged >40 years). In order to evaluate the time course of putative changes in BNP response in TTC, we correlated duration of symptoms (from the time of onset of symptoms till blood sampling). Also, correlations were sought between plasma NT-proBNP levels, platelet responsiveness to SNP, patients' inflammation status, LVEF, pulmonary capillary wedge pressure (PCWP), systolic blood pressure on admission, catecholamine release and impact of angiotensin converting enzyme (ACE) inhibitors and residual BNP response.

3.5.3 Results

3.5.3.1 Patient characteristics

The clinical characteristics of the 19 acute TTC patients are summarized in Table 3-2. All patients were female, aged >40 years, and none had evidence of pulmonary congestion although LV systolic function varied substantially.

Evaluation occurred 0.5 to 5.5 days post onset of symptoms: this corresponded to marked but variable evaluation of NT-proBNP and hs-CRP, with 6 of the 19 patients having started treatment with ACE inhibitors at the time of blood sampling.

Table 3-2: Clinical characteristics of TTC

TTC patients	*N=19*
Age (years±SEM)	70±2
Sex (M:F)	0:19
Peak troponin (ng/L)	441±62
LVEF (%)	44±2
T2 score (median) #	0.65
Normetanephrine (pmol/L, median)	1230
Metanephrine (pmol/L, median)	230
Peak NT-proBNP (pg/mL, median)	4569
hs-CRP (mg/L, median)	7.2
ACE inhibitor therapy (n/%)	6/32

Method of (Neil, C et al. 2012); Normal range: 0.47±0.04 arbitrary units

3.5.3.2 Effects of BNP on neutrophil O_2^- generation in TTC patients

As regards the impact of BNP in suppressing PMA- and fMLP-induced O_2^- release in TTC patients, significant attenuation was observed compared to control subjects (Figure 3-14): - in general, there was no suppression of O_2^- release in the TTC population, but there was considerable inter-individual variability.

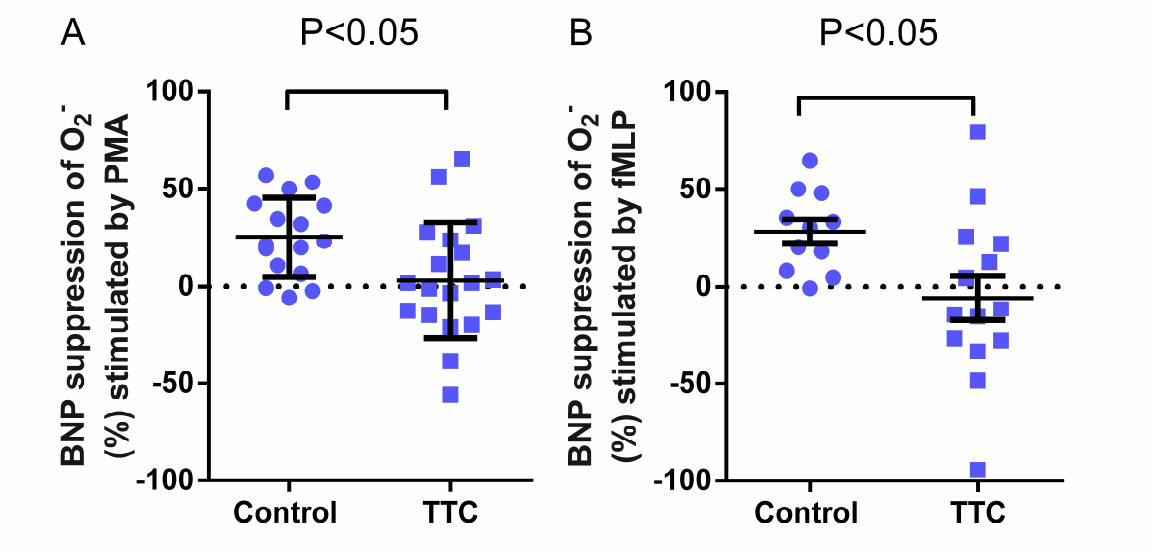

Figure 3-14: Comparison of BNP effects on neutrophils O_2^- generation between control subjects and TTC patients.

*(A) BNP effects on PMA-induced O_2^- generation: Control (n=16), TTC patients (n=19). *P<0.05.*

*(B) BNP effects on fMLP-induced O_2^- generation: Control (n=11), TTC patients (n=13). *P<0.05.*

The age of the TTC cohort was significantly greater than that of the control subjects (mean ages 70±2 and 58±2 years respectively, P<0.01). Furthermore, only 69% of the control

subjects were females. Of the control subjects, 9 were age-matched females (mean age 60±3 years). In these subjects, BNP induced similar suppression of O_2^- release to the entire control group (20±7 % for PMA-stimulation; and 30±8 % for fMLP-stimulation).

3.5.3.3 Correlations of clinical parameters with BNP effects

Among TTC patients, no correlation was observed between metanephrine, normetanephrine, hs-CRP (Figure 3-15), LVEF, T2 score, troponin T, peak NT-proBNP levels (Figure 3-16) and BNP response; furthermore duration of symptoms (2.5±0.3 days) was also not a significant determinant of BNP response (Figure 3-17); moreover, unlike controls, platelet responsiveness to SNP was not correlated with the residual BNP response in TTC patients (Figure 3-18).

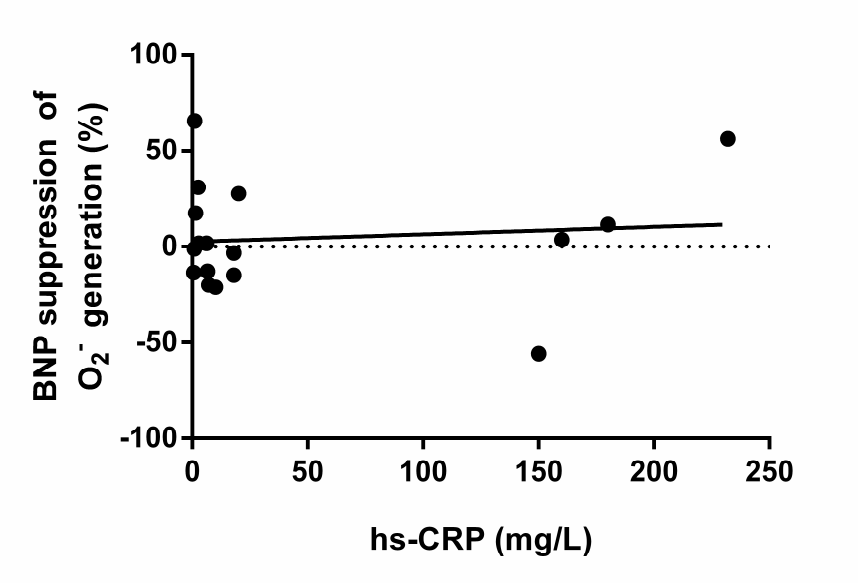

Figure 3-15: Correlation between hs-CRP levels and extent of BNP effects on neutrophil O_2^- generation.

r=0.1, P=NS.

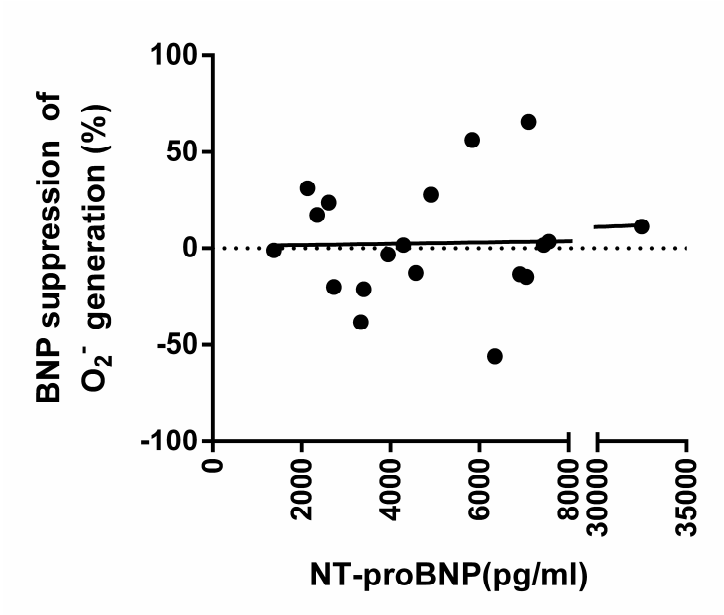

Figure 3-16: Correlation between plasma NT-proBNP levels and extent of BNP effects on neutrophil O_2^- generation.

r=0.08, P=NS.

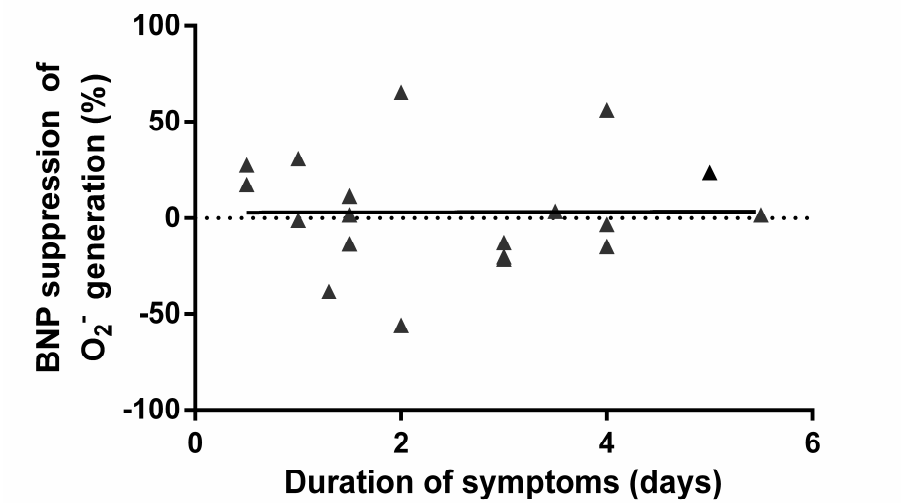

Figure 3-17: Correlation between duration of onset symptoms and extent of BNP effects on neutrophil O_2^- generation.

r=0.004, P=NS.

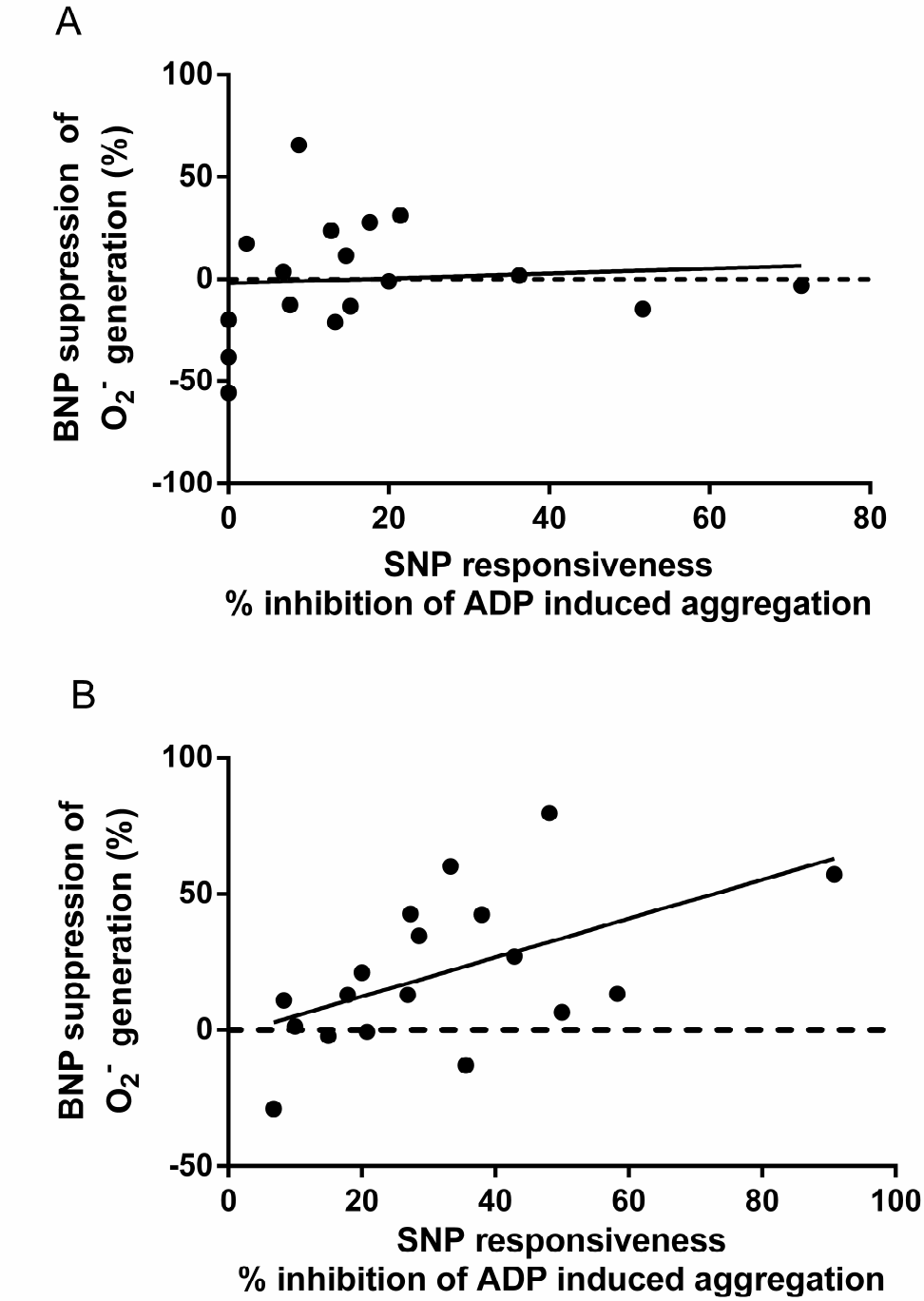

Figure 3-18: Correlations between BNP effects in isolated neutrophils and SNP responsiveness in whole blood.

(A) TTC patients (r=0.08, P=NS); (B) Healthy control subjects (r=0.54, P=0.02).

3.5.3.4 Impact of treatment of TTC

Acute TTC patients, 5 in whom initial BNP response was blunted were re-approached after at least 3 months recovery. The NT-proBNP level was 206pg/mL (median), which is significantly decreased compared to peak levels in acute phase. However, no improvement was observed with neutrophil response to BNP (Figure 3-19).

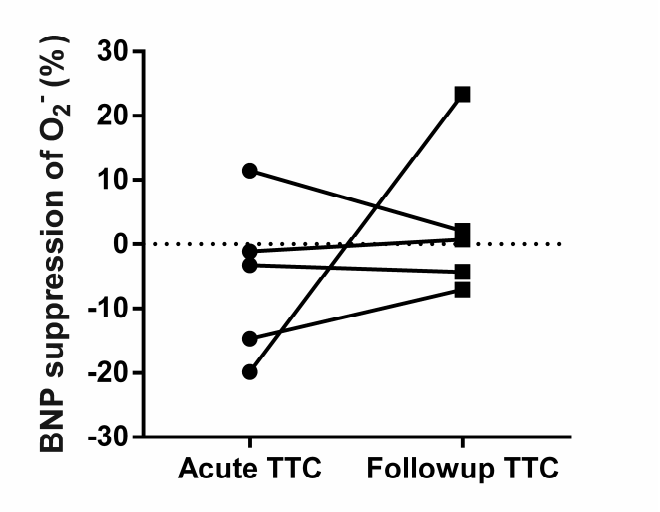

Figure 3-19: Comparison of BNP effects on neutrophil O_2^- generation in response to PMA between acute and follow up TTC patients.

3.5.4 Discussion

In this pilot study we found that the suppressing of O_2^- generation effect of BNP is attenuated in patients in the acute stages of TTC, in association with marked increases of plasma levels of NT-proBNP. Thus, TTC, like acute HF, is associated with suppression of BNP effect.

The patients concerned were studied more than 12 hours after the onset of symptoms, at which time there was no correlation between plasma NT-proBNP levels and residual BNP

effect. Thus the study did not reveal: (1) The time course for suppression of BNP effect; (2) The extent of BNP release necessary to induce such suppression. However, the results of this pilot study can be combined with those of the experiment described in chapter 3.4 to infer that suppression of BNP effect is likely to result from BNP release per se, rather than from associated redox stress.

Chan et al. (Chan et al. 2012) demonstrated that both BNP-induced and NO-induced release of cGMP could under some circumstances perpetuate increased catecholamine release. It remains to be seen whether progressive attenuation of BNP-based signaling in TTC functions in part to "turn off" the potential vicious cycle of associated cGMP release and continuing catecholamine effects on the myocardium.

Although the molecular mechanisms involved in TTC pathophysiology have not been fully defined, the fact that inflammatory processes induce cardiac damage is clear. Recent studies demonstrated that TTC is associated with intense myocardial inflammation (Eitel et al. 2010; Nef et al. 2007; Yoshida et al. 2007) and "inflammatory" BNP release (Akashi et al. 2004; Grabowski et al. 2008; Nef et al. 2008; Nguyen et al. 2011), which is a different mechanism of BNP release compared to that in HF patients (in which the elevated BNP levels is predominantly due to increased ventricular stress). Paradoxically, this could result in rapid suppression of the BNP-mediated signal transduction, and thus perpetuation of the inflammatory response.

The data on recovery of BNP signaling are only fragmentary, but even with n=5 patients it appears that a considerable period of time elapses before normal function is restored after TTC. Interestingly, this was despite the fact that NT-proBNP levels had returned almost to normal values. It is possible that factors other than extent of BNP elevation, such as redox stress, contribute to down-regulation of BNP signaling in this circumstance.

Although this pilot study establishes that BNP-based anti-inflammatory signaling is attenuated in TTC, it leaves several key questions unanswered. First, does this attenuation also apply to the vasodilator effects of BNP, which have been postulated to play a role in the early hypotension and shock seen in some patients (Chong et al. 2013). Second, does BNP signaling recover? In general, BNP levels fall progressively over the first 3 months post onset of TTC (Nguyen et al. 2011) and restoration of BNP-based anti-inflammatory effects might be relevant to the eventual resolution of symptoms. However, the data on follow-up patients were very limited. Finally, is suppression of BNP effect "inevitable" for all conditions where BNP levels are markedly elevated for any length of time? If this is the case, the homeostatic role of BNP is actually a very limited one.

Chapter 4: Summary, conclusions and future perspectives:

Although extensive research efforts have been made in the past decades, cardiovascular disease, together with cancer, remains the most important cause of death in Western societies (Stewart et al. 2001), and the underlying pathophysiological mechanisms remain incompletely understood. B-type natriuretic peptide (BNP) is a well-known endogenous vasodilator, which also exerts natriuretic and anti-hypertrophic effects and improves left ventricular (LV) relaxation (Potter, Abbey-Hosch & Dickey 2006). As LV distension in heart failure (HF) stimulates BNP release, this could be expected to restore cardiovascular homeostasis, as should exogenous administration of BNP. However, clinical data suggest that HF may be associated with tissue resistance to BNP (O'Connor et al. 2011), and it is possible that this resistance represents the main limitation to efficacy of BNP-based therapeutics.

The objective of the current PhD project was to address a number of issues regarding attenuated BNP effects in cardiovascular diseases. A state of prolonged exposure to high concentrations of BNP/NT-proBNP both in HF and Tako-tsubo cardiomyopathy (TTC) patients might theoretically be associated with BNP receptor desensitization or internalization.

This thesis encompasses three studies designed to address the possibility of attenuated BNP effects in cardiovascular disease. The first part of the thesis describes studies performed in isolated human neutrophils obtained from healthy subjects, focusing on BNP effect on the neutrophil burst, an effect which was evaluated when it became clear that BNP stimulation induced too little release of cGMP for routine quantitation. The second part focuses on the effects of BNP on neutrophil superoxide (O_2^-) generation in patients

with HF, both acute and chronic. The third part of the experimental studies was performed in TTC patients to examine whether the markedly elevated circulating BNP levels desensitize BNP response.

1) In control subjects (without diagnosed cardiovascular disease), we have identified a novel effect of BNP in suppressing the release of the inflammatory modulator O_2^- from isolated neutrophils. This effect of BNP is likely to be mediated by the cGMP-PKG pathway, because BNP effect is mimicked by a cGMP analogue and abolished by a PKG inhibitor.

Although neutrophil O_2^- generation has long been recognized as an important component of host defense systems to protect the body from the injuries of microbial organisms, it plays a pivotal role in the pathophysiology of cardiovascular disease as an important source of reactive oxygen species (ROS) generation. The suppressing effect of BNP on neutrophil O_2^- generation might have clinical importance as BNP has beneficial effects on myocardial ischemia-reperfusion injury (Burley & Baxter 2007; Hu et al. 2014; Ren et al. 2010; Sun, YG et al. 2010; Wu, B et al. 2009), by reducing the expression of pro-inflammatory cytokines, apoptosis, myocardial kinases and thus limiting infarct size. The next issue, therefore, is whether this potentially beneficial effect is maintained in the presence of incremental BNP release, as in acute (and chronic) HF: if so, BNP-based therapy might be useful in limiting inflammatory responses.

The O_2^- suppressing effect currently demonstrated with BNP is in contrast to previous studies which have reported priming effects of A-type natriuretic peptide and BNP on neutrophil O_2^- release (Garlichs et al. 1999; Wiedermann et al. 1992). However, the O_2^- suppressing effect is analogous to that seen in previously studies utilizing nitric oxide

donors, which are known to activate soluble guanlyly cyclase to produce cGMP (Ervens, Schultz & Seifert 1991; Moilanen et al. 1993; Wenzel-Seifert, Ervens & Seifert 1991).

2) The effects of BNP in suppressing O_2^- generation are significantly attenuated in patients with acute HF, but recover partially with chronic treatment of HF: - better recovery tends to occur in younger patients. Furthermore, the cGMP analog effect on neutrophil O_2^- generation in acute HF patients is similar to that in control subjects.

"BNP resistance" has been described in a number of studies (Baerts et al. 2012; Nakamura, M et al. 1998; Tsutamoto, Wada, Maeda, Hisanaga, Maeda, et al. 1997). The finding in the present study extends this conclusion, thus explaining the ineffectiveness of Nesiritide as a HF treatment. The attenuated BNP effects might also contribute to perpetuation of inflammatory activation in acute HF patients. Circulating "BNP" measured by current methods, might not entirely reflect the active BNP1-32 (Liang et al. 2007): - other forms of BNP fragments have been identified as well as increased activity of BNP degradation enzymes (Boerrigter et al. 2009; Boerrigter et al. 2007; Brandt et al. 2006; Muller et al. 1992; Pankow et al. 2007; Ralat et al. 2011; Toll et al. 1991). Understanding of BNP biology and physiology is still limited, and more information regarding its processing, bioactivity and clearance in different disease states is required to enable the effects of BNP to be utilized optimally for HF management.

3) In the acute stage of TTC, suppression of O_2^- generation effect by BNP is attenuated, in association but not in proportion with marked increases of plasma levels of NT-proBNP. This might be particularly important in TTC, where both BNP elevation (Nguyen et al. 2011) and myocardial inflammation (Neil, C et al. 2012) have been shown to persist for at least 3 months after acute attacks. Potentially therapeutic restoration of BNP effect might accelerate recovery.

The results of the current study demonstrating attenuated BNP effects in acute TTC, with marked elevated BNP levels, suggest that this is a "general" response to elevated levels. While the beneficial effects of BNP are lost, any residual BNP-induced cGMP formation might promote catecholamine release (Chan et al. 2012) and further harm the myocardium in TTC patients.

The studies described in this thesis therefore add another aspect to the understanding of BNP effects in HF patients and may guide the development for the pharmacotherapy of HF. A number of residual issues should be addressed in future research studies. Specifically mechanistic studies on attenuated BNP responsiveness in cardiac tissue should be performed in animal model. Furthermore, BNP effect on reducing inflammation should be studied in cardiomyocytes. It should not be assumed that oxidative stress within myocardium is of indirect "infiltrative" origin: the main source of cardiac ROS is myocardial cells. Moreover, hypotension is one of the side effects for Nesiritide therapy, and BNP exerts vasodilatory effects as well: - therefore, evaluating vascular responses to BNP in HF settings is essential to understand the role of BNP in the pathophysiology of HF. BNP responsiveness should also be evaluated with different circulating BNP levels to work out whether there is a correlation between BNP response and BNP levels. Furthermore, it will be interesting to examine the BNP effects in recovered TTC patients.

Finally, the precise nexus between integrity of BNP signaling and suppression of neutrophil O_2^- release is of further interest. The attenuation of a biochemical response to BNP, whether it is purely cGMP production or O_2^- suppression, presumably reflects either down-regulation or internalization of natriuretic peptide receptors. The mechanics of this process have not been evaluated to date. It is even possible that oxidative stress engendered by O_2^- release might predispose towards down-regulation or internalization of these

receptors, contributing to the potential for a “vicious cycle” effect in acute HF. The highest priority should therefore be accorded to elucidating these mechanisms.

References

Abadeh, S, Case, PC & Harrison, R 1993, 'Purification of xanthine oxidase from human heart', *Biochem Soc Trans*, vol. 21, no. 2, May, p. 99S.

Abraham, WT, Lowes, BD, Ferguson, DA, Odom, J, Kim, JK, Robertson, AD, Bristow, MR & Schrier, RW 1998, 'Systemic hemodynamic, neurohormonal, and renal effects of a steady-state infusion of human brain natriuretic peptide in patients with hemodynamically decompensated heart failure', *J Card Fail*, vol. 4, no. 1, Mar, pp. 37-44.

Abraham, WT, Trupp, RJ & Jarjoura, D 2010, 'Nesiritide in acute decompensated heart failure: a pooled analysis of randomized controlled trials', *Clin Cardiol*, vol. 33, no. 8, Aug, pp. 484-489.

Adamopoulos, S, Parissis, JT & Kremastinos, DT 2001, 'A glossary of circulating cytokines in chronic heart failure', *Eur J Heart Fail*, vol. 3, no. 5, Oct, pp. 517-526.

Ago, T, Kuroda, J, Pain, J, Fu, C, Li, H & Sadoshima, J 2010, 'Upregulation of Nox4 by hypertrophic stimuli promotes apoptosis and mitochondrial dysfunction in cardiac myocytes', *Circ Res*, vol. 106, no. 7, Apr 16, pp. 1253-1264.

Ago, T, Matsushima, S, Kuroda, J, Zablocki, D, Kitazono, T & Sadoshima, J 2010, 'The NADPH oxidase Nox4 and aging in the heart', *Aging (Albany NY)*, vol. 2, no. 12, Dec, pp. 1012-1016.

AIHW 2010, *Australia's health 2010. Australia's health no. 12. Cat. no. AUS 122*, Canberra, AIHW, viewed 8 June 2014, <http://www.aihw.gov.au/publication-detail/?id=6442468376>.

AIHW 2012, *Australia's health 2012. Australia's health no. 13. Cat. no. AUS 156*, Canberra: AIHW, viewed 8 June 2014, <http://www.aihw.gov.au/publication-detail/?id=10737422172>.

Akashi, YJ, Musha, H, Nakazawa, K & Miyake, F 2004, 'Plasma brain natriuretic peptide in takotsubo cardiomyopathy', *QJM*, vol. 97, no. 9, Sep, pp. 599-607.

Akashi, YJ, Nakazawa, K, Sakakibara, M, Miyake, F, Koike, H & Sasaka, K 2003, 'The clinical features of takotsubo cardiomyopathy', *QJM*, vol. 96, no. 8, Aug, pp. 563-573.

Aker, S, Belosjorow, S, Konietzka, I, Duschin, A, Martin, C, Heusch, G & Schulz, R 2003, 'Serum but not myocardial TNF-alpha concentration is increased in pacing-induced heart failure in rabbits', *Am J Physiol Regul Integr Comp Physiol*, vol. 285, no. 2, Aug, pp. R463-469.

Ala-Kopsala, M, Moilanen, AM, Rysa, J, Ruskoaho, H & Vuolteenaho, O 2010, 'Characterization of molecular forms of N-terminal B-type natriuretic peptide in vitro', *Clin Chem*, vol. 56, no. 12, Dec, pp. 1822-1829.

Alderton, WK, Cooper, CE & Knowles, RG 2001, 'Nitric oxide synthases: structure, function and inhibition', *Biochem J*, vol. 357, no. Pt 3, Aug 1, pp. 593-615.

Amir, O, Paz, H, Rogowski, O, Barshai, M, Sagiv, M, Shnizer, S, Reznick, AZ & Amir, RE 2009, 'Serum oxidative stress level correlates with clinical parameters in chronic systolic heart failure patients', *Clin Cardiol*, vol. 32, no. 4, Apr, pp. 199-203.

Anand, IS, Gurden, J, Wander, GS, Ogara, P, Harding, SE, Ferrari, R, Cornacchiari, A, Panzali, A, Wahi, PL & Poolewilson, PA 1991, 'Cardiovascular and Hormonal Effects of Calcitonin Gene-Related Peptide in Congestive-Heart-Failure', *Journal of the American College of Cardiology*, vol. 17, no. 1, Jan, pp. 208-217.

Anand, IS, Latini, R, Florea, VG, Kuskowski, MA, Rector, T, Masson, S, Signorini, S, Mocarelli, P, Hester, A, Glazer, R, Cohn, JN & Val-He, FTI 2005, 'C-reactive protein in heart failure: prognostic value and the effect of valsartan', *Circulation*, vol. 112, no. 10, Sep 6, pp. 1428-1434.

Anatoliotakis, N, Deftereos, S, Bouras, G, Giannopoulos, G, Tsounis, D, Angelidis, C, Kaoukis, A & Stefanadis, C 2013, 'Myeloperoxidase: expressing inflammation and oxidative stress in cardiovascular disease', *Curr Top Med Chem*, vol. 13, no. 2, pp. 115-138.

Anderson, RA, Ellis, GR, Chirkov, YY, Holmes, AS, Payne, N, Blackman, DJ, Jackson, SK, Lewis, MJ, Horowitz, JD & Frenneaux, MP 2004, 'Determinants of platelet responsiveness to nitric oxide in patients with chronic heart failure', *Eur J Heart Fail*, vol. 6, no. 1, Jan, pp. 47-54.

Andreassi, MG, Del Ry, S, Palmieri, C, Clerico, A, Biagini, A & Giannessi, D 2001, 'Up-regulation of 'clearance' receptors in patients with chronic heart failure: a possible explanation for the resistance to biological effects of cardiac natriuretic hormones', *Eur J Heart Fail*, vol. 3, no. 4, Aug, pp. 407-414.

Anker, SD, Chua, TP, Ponikowski, P, Harrington, D, Swan, JW, Kox, WJ, Poole-Wilson, PA & Coats, AJ 1997, 'Hormonal changes and catabolic/anabolic imbalance in chronic heart failure and their importance for cardiac cachexia', *Circulation*, vol. 96, no. 2, Jul 15, pp. 526-534.

Arora, RR, Venkatesh, PK & Molnar, J 2006, 'Short and long-term mortality with nesiritide', *Am Heart J*, vol. 152, no. 6, Dec, pp. 1084-1090.

Arruda-Olson, AM, Reeder, GS, Bell, MR, Weston, SA & Roger, VL 2009, 'Neutrophilia predicts death and heart failure after myocardial infarction: a community-based study', *Circ Cardiovasc Qual Outcomes*, vol. 2, no. 6, Nov, pp. 656-662.

Atlas, SA 2007, 'The renin-angiotensin aldosterone system: pathophysiological role and pharmacologic inhibition', *J Manag Care Pharm*, vol. 13, no. 8 Suppl B, Oct, pp. 9-20.

Aukrust, P, Ueland, T, Lien, E, Bendtzen, K, Muller, F, Andreassen, AK, Nordoy, I, Aass, H, Espevik, T, Simonsen, S, Froland, SS & Gullestad, L 1999, 'Cytokine network in congestive heart failure secondary to ischemic or idiopathic dilated cardiomyopathy', *Am J Cardiol*, vol. 83, no. 3, Feb 1, pp. 376-382.

Aukrust, P, Ueland, T, Muller, F, Andreassen, AK, Nordoy, I, Aas, H, Kjekshus, J, Simonsen, S, Froland, SS & Gullestad, L 1998, 'Elevated circulating levels of C-C chemokines in patients with congestive heart failure', *Circulation*, vol. 97, no. 12, Mar 31, pp. 1136-1143.

Babior, BM 1978, 'Oxygen-dependent microbial killing by phagocytes (first of two parts)', *N Engl J Med*, vol. 298, no. 12, Mar 23, pp. 659-668.

Babior, BM 1999, 'NADPH oxidase: an update', *Blood*, vol. 93, no. 5, Mar 1, pp. 1464-1476.

Babior, BM, Lambeth, JD & Nauseef, W 2002, 'The neutrophil NADPH oxidase', *Arch Biochem Biophys*, vol. 397, no. 2, Jan 15, pp. 342-344.

Badwey, JA & Karnovsky, ML 1980, 'Active oxygen species and the functions of phagocytic leukocytes', *Annu Rev Biochem*, vol. 49, pp. 695-726.

Baerts, L, Gomez, N, Vanderheyden, M, De Meester, I & Mc Entee, K 2012, 'Possible mechanisms for brain natriuretic peptide resistance in heart failure with a focus on interspecies differences and canine BNP biology', *Vet J*, vol. 194, Sep 27, pp. 34-39.

Balligand, JL, Kobzik, L, Han, XQ, Kaye, DM, Belhassen, L, Ohara, DS, Kelly, RA, Smith, TW & Michel, T 1995, 'Nitric Oxide-Dependent Parasympathetic Signaling Is Due to Activation of Constitutive Endothelial (Type-Iii) Nitric-Oxide Synthase in Cardiac Myocytes', *Journal of Biological Chemistry*, vol. 270, no. 24, Jun 16, pp. 14582-14586.

Balligand, JL, Ungureanulongrois, D, Simmons, WW, Pimental, D, Malinski, TA, Kapturczak, M, Taha, Z, Lowenstein, CJ, Davidoff, AJ, Kelly, RA, Smith, TW & Michel, T 1994, 'Cytokine-Inducible Nitric-Oxide Synthase (Inos) Expression in Cardiac Myocytes - Characterization and Regulation of Inos Expression and Detection of Inos Activity in Single Cardiac Myocytes in-Vitro', *Journal of Biological Chemistry*, vol. 269, no. 44, Nov 4, pp. 27580-27588.

Bannister, JV, Bellavite, P, Serra, MC, Thornalley, PJ & Rossi, F 1982, 'An EPR study of the production of superoxide radicals by neutrophil NADPH oxidase', *FEBS Lett*, vol. 145, no. 2, Aug 23, pp. 323-326.

Bauersachs, J, Bouloumie, A, Fraccarollo, D, Hu, K, Busse, R & Ertl, G 1999, 'Endothelial dysfunction in chronic myocardial infarction despite increased vascular endothelial nitric oxide synthase and soluble guanylate cyclase expression: role of enhanced vascular superoxide production', *Circulation*, vol. 100, no. 3, Jul 20, pp. 292-298.

Baumgarten, W 1898, 'Infarction in the Heart', *J Boston Soc Med Sci*, vol. 3, no. 2, Nov 18, pp. 39-40.

Beckmann, JS, Ye, YZ, Anderson, PG, Chen, J, Accavitti, MA, Tarpey, MM & White, CR 1994, 'Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry', *Biol Chem Hoppe Seyler*, vol. 375, no. 2, Feb, pp. 81-88.

Belch, JJ, Bridges, AB, Scott, N & Chopra, M 1991, 'Oxygen free radicals and congestive heart failure', *Br Heart J*, vol. 65, no. 5, May, pp. 245-248.

Bellamy, J, Bowen, EJ, Russo, AF & Durham, PL 2006, 'Nitric oxide regulation of calcitonin gene-related peptide gene expression in rat trigeminal ganglia neurons', *Eur J Neurosci*, vol. 23, no. 8, Apr, pp. 2057-2066.

Benites-Zapata, VA, Hernandez, AV, Nagarajan, V, Cauthen, CA, Starling, RC & Tang, WH 2015, 'Usefulness of Neutrophil-to-Lymphocyte Ratio in Risk Stratification of Patients With Advanced Heart Failure', *Am J Cardiol*, vol. 115, no. 1, Jan 1, pp. 57-61.

Boerrigter, G, Costello-Boerrigter, LC, Harty, GJ, Huntley, BK, Cataliotti, A, Lapp, H & Burnett, JC, Jr. 2009, 'B-type natriuretic peptide 8-32, which is produced from mature BNP 1-32 by the metalloprotease meprin A, has reduced bioactivity', *Am J Physiol Regul Integr Comp Physiol*, vol. 296, no. 6, Jun, pp. R1744-1750.

Boerrigter, G, Costello-Boerrigter, LC, Harty, GJ, Lapp, H & Burnett, JC, Jr. 2007, 'Des-serine-proline brain natriuretic peptide 3-32 in cardiorenal regulation', *Am J Physiol Regul Integr Comp Physiol*, vol. 292, no. 2, Feb, pp. R897-901.

Bonetti, PO, Lerman, LO & Lerman, A 2003, 'Endothelial dysfunction: a marker of atherosclerotic risk', *Arterioscler Thromb Vasc Biol*, vol. 23, no. 2, Feb 1, pp. 168-175.

Boyum, A 1968, 'Isolation of leucocytes from human blood. Further observations. Methylcellulose, dextran, and ficoll as erythrocyteaggregating agents', *Scand J Clin Lab Invest Suppl*, vol. 97, pp. 31-50.

Brain, SD, MacIntyre, I & Williams, TJ 1986, 'A second form of human calcitonin gene-related peptide which is a potent vasodilator', *Eur J Pharmacol*, vol. 124, no. 3, May 27, pp. 349-352.

Brain, SD & Williams, TJ 1985, 'Inflammatory Edema Induced by Synergism between Calcitonin Gene-Related Peptide (Cgrp) and Mediators of Increased Vascular-Permeability', *British Journal of Pharmacology*, vol. 86, no. 4, pp. 855-860.

Brandt, I, Lambeir, AM, Ketelslegers, JM, Vanderheyden, M, Scharpe, S & De Meester, I 2006, 'Dipeptidyl-peptidase IV converts intact B-type natriuretic peptide into its des-SerPro form', *Clin Chem*, vol. 52, no. 1, Jan, pp. 82-87.

Braunwald, E 2008, 'Biomarkers in heart failure', *N Engl J Med*, vol. 358, no. 20, May 15, pp. 2148-2159.

Braunwald, E 2013, 'Heart Failure', *JACC Heart Fail*, vol. 1, no. 1, Feb, pp. 1-20.

Brewster, UC & Perazella, MA 2004, 'The renin-angiotensin-aldosterone system and the kidney: effects on kidney disease', *Am J Med*, vol. 116, no. 4, Feb 15, pp. 263-272.

Bristow, MR, Ginsburg, R, Minobe, W, Cubicciotti, RS, Sageman, WS, Lurie, K, Billingham, ME, Harrison, DC & Stinson, EB 1982, 'Decreased catecholamine sensitivity and beta-adrenergic-receptor density in failing human hearts', *N Engl J Med*, vol. 307, no. 4, Jul 22, pp. 205-211.

Brown, GE, Stewart, MQ, Bissonnette, SA, Elia, AE, Wilker, E & Yaffe, MB 2004, 'Distinct ligand-dependent roles for p38 MAPK in priming and activation of the neutrophil NADPH oxidase', *J Biol Chem*, vol. 279, no. 26, Jun 25, pp. 27059-27068.

Bryan, PM, Xu, X, Dickey, DM, Chen, Y & Potter, LR 2007, 'Renal hyporesponsiveness to atrial natriuretic peptide in congestive heart failure results from reduced atrial natriuretic peptide receptor concentrations', *Am J Physiol Renal Physiol*, vol. 292, no. 5, May, pp. F1636-1644.

Bryk, R, Griffin, P & Nathan, C 2000, 'Peroxynitrite reductase activity of bacterial peroxiredoxins', *Nature*, vol. 407, no. 6801, Sep 14, pp. 211-215.

Burley, DS & Baxter, GF 2007, 'B-type natriuretic peptide at early reperfusion limits infarct size in the rat isolated heart', *Basic Research in Cardiology*, vol. 102, no. 6, Nov, pp. 529-541.

Bybee, KA, Kara, T, Prasad, A, Lerman, A, Barsness, GW, Wright, RS & Rihal, CS 2004, 'Systematic review: transient left ventricular apical ballooning: a syndrome that mimics ST-segment elevation myocardial infarction', *Ann Intern Med*, vol. 141, no. 11, Dec 7, pp. 858-865.

Cappola, TP, Kass, DA, Nelson, GS, Berger, RD, Rosas, GO, Kobeissi, ZA, Marban, E & Hare, JM 2001, 'Allopurinol improves myocardial efficiency in patients with idiopathic dilated cardiomyopathy', *Circulation*, vol. 104, no. 20, Nov 13, pp. 2407-2411.

Cardinal, DC & Flower, RJ 1980, 'The electronic aggregometer: a novel device for assessing platelet behavior in blood', *J Pharmacol Methods*, vol. 3, no. 2, Feb, pp. 135-158.

Casals, G, Ros, J, Sionis, A, Davidson, MM, Morales-Ruiz, M & Jimenez, W 2009, 'Hypoxia induces B-type natriuretic peptide release in cell lines derived from human cardiomyocytes', *Am J Physiol Heart Circ Physiol*, vol. 297, no. 2, Aug, pp. H550-555.

Chamseddine, AH & Miller, FJ, Jr. 2003, 'Gp91phox contributes to NADPH oxidase activity in aortic fibroblasts but not smooth muscle cells', *Am J Physiol Heart Circ Physiol*, vol. 285, no. 6, Dec, pp. H2284-2289.

Chan, NY, Seyedi, N, Takano, K & Levi, R 2012, 'An unsuspected property of natriuretic peptides: promotion of calcium-dependent catecholamine release via protein kinase G-mediated phosphodiesterase type 3 inhibition', *Circulation*, vol. 125, no. 2, Jan 17, pp. 298-307.

Chandra, A, Otero, R, Freeman, D & Cairns, CB 2008, 'BNP-mediated vasodilatation for dialysis-dependent patient with acute heart failure syndrome in the emergency department', *Ren Fail*, vol. 30, no. 1, pp. 45-50.

Chang, T & Wu, L 2006, 'Methylglyoxal, oxidative stress, and hypertension', *Can J Physiol Pharmacol*, vol. 84, no. 12, Dec, pp. 1229-1238.

Charles, CJ, Espiner, EA, Nicholls, MG, Richards, AM, Yandle, TG, Protter, A & Kosoglou, T 1996, 'Clearance receptors and endopeptidase 24.11: equal role in natriuretic peptide metabolism in conscious sheep', *Am J Physiol*, vol. 271, no. 2 Pt 2, Aug, pp. R373-380.

Charniot, JC, Vignat, N, Albertini, JP, Bogdanova, V, Zerhouni, K, Monsuez, JJ, Legrand, A, Artigou, JY & Bonnefont-Rousselot, D 2008, 'Oxidative stress in patients with acute heart failure', *Rejuvenation Res*, vol. 11, no. 2, Apr, pp. 393-398.

Chen, HH 2007, 'Heart failure: a state of brain natriuretic peptide deficiency or resistance or both!', *J Am Coll Cardiol*, vol. 49, no. 10, Mar 13, pp. 1089-1091.

Chen, HH, Glockner, JF, Schirger, JA, Cataliotti, A, Redfield, MM & Burnett, JC, Jr. 2012, 'Novel protein therapeutics for systolic heart failure: chronic subcutaneous B-type natriuretic peptide', *J Am Coll Cardiol*, vol. 60, no. 22, Dec 4, pp. 2305-2312.

Cheng, V, Kazanagra, R, Garcia, A, Lenert, L, Krishnaswamy, P, Gardetto, N, Clopton, P & Maisel, A 2001, 'A rapid bedside test for B-type peptide predicts treatment outcomes in patients admitted for decompensated heart failure: a pilot study', *J Am Coll Cardiol*, vol. 37, no. 2, Feb, pp. 386-391.

Chidsey, CA, Harrison, DC & Braunwald, E 1962, 'Augmentation of the plasma nor-epinephrine response to exercise in patients with congestive heart failure', *N Engl J Med*, vol. 267, Sep 27, pp. 650-654.

Chirkov, YY, Holmes, AS, Chirkova, LP & Horowitz, JD 1999, 'Nitrate resistance in platelets from patients with stable angina pectoris', *Circulation*, vol. 100, no. 2, Jul 13, pp. 129-134.

Chirkov, YY & Horowitz, JD 2007, 'Impaired tissue responsiveness to organic nitrates and nitric oxide: a new therapeutic frontier?', *Pharmacol Ther*, vol. 116, no. 2, Nov, pp. 287-305.

Chong, CR, Neil, CJ, Nguyen, TH, Stansborough, J, Law, GW, Singh, K & Horowitz, JD 2013, 'Dissociation between severity of takotsubo cardiomyopathy and presentation with shock or hypotension', *Clin Cardiol*, vol. 36, no. 7, Jul, pp. 401-406.

Cifuentes-Pagano, E, Csanyi, G & Pagano, PJ 2012, 'NADPH oxidase inhibitors: a decade of discovery from Nox2ds to HTS', *Cell Mol Life Sci*, vol. 69, no. 14, Jul, pp. 2315-2325.

Clancy, RM, Leszczynska-Piziak, J & Abramson, SB 1992, 'Nitric oxide, an endothelial cell relaxation factor, inhibits neutrophil superoxide anion production via a direct action on the NADPH oxidase', *J Clin Invest*, vol. 90, no. 3, Sep, pp. 1116-1121.

Cleland, JG, Dargie, HJ & Ford, I 1987, 'Mortality in heart failure: clinical variables of prognostic value', *Br Heart J*, vol. 58, no. 6, Dec, pp. 572-582.

Clerico, A, Iervasi, G, Del Chicca, MG, Emdin, M, Maffei, S, Nannipieri, M, Sabatino, L, Forini, F, Manfredi, C & Donato, L 1998, 'Circulating levels of cardiac natriuretic peptides (ANP and BNP) measured by highly sensitive and specific immunoradiometric assays in normal subjects and in patients with different degrees of heart failure', *J Endocrinol Invest*, vol. 21, no. 3, Mar, pp. 170-179.

Cohn, JN, Levine, TB, Olivari, MT, Garberg, V, Lura, D, Francis, GS, Simon, AB & Rector, T 1984, 'Plasma norepinephrine as a guide to prognosis in patients with chronic congestive heart failure', *N Engl J Med*, vol. 311, no. 13, Sep 27, pp. 819-823.

Colombo, PC, Banchs, JE, Celaj, S, Talreja, A, Lachmann, J, Malla, S, DuBois, NB, Ashton, AW, Latif, F, Jorde, UP, Ware, JA & LeJemtel, TH 2005, 'Endothelial cell activation in patients with decompensated heart failure', *Circulation*, vol. 111, no. 1, Jan 4, pp. 58-62.

Colucci, WS, Elkayam, U, Horton, DP, Abraham, WT, Bourge, RC, Johnson, AD, Wagoner, LE, Givertz, MM, Liang, CS, Neibaur, M, Haught, WH & LeJemtel, TH 2000, 'Intravenous nesiritide, a natriuretic peptide, in the treatment of decompensated congestive heart failure. Nesiritide Study Group', *N Engl J Med*, vol. 343, no. 4, Jul 27, pp. 246-253.

Comini, L, Bachetti, T, Gaia, G, Pasini, E, Agnoletti, L, Pepi, P, Ceconi, C, Curello, S & Ferrari, R 1996, 'Aorta and skeletal muscle NO synthase expression in experimental heart failure', *J Mol Cell Cardiol*, vol. 28, no. 11, Nov, pp. 2241-2248.

Conraads, VM, Bosmans, JM, Schuerwegh, AJ, Goovaerts, I, De Clerck, LS, Stevens, WJ, Bridts, CH & Vrints, CJ 2005, 'Intracellular monocyte cytokine production and CD 14

expression are up-regulated in severe vs mild chronic heart failure', *J Heart Lung Transplant*, vol. 24, no. 7, Jul, pp. 854-859.

Cotton, JM, Kearney, MT & Shah, AM 2002, 'Nitric oxide and myocardial function in heart failure: friend or foe?', *Heart*, vol. 88, no. 6, Dec, pp. 564-566.

Cowie, MR & Mendez, GF 2002, 'BNP and congestive heart failure', *Prog Cardiovasc Dis*, vol. 44, no. 4, Jan-Feb, pp. 293-321.

Cowie, MR, Wood, DA, Coats, AJ, Thompson, SG, Suresh, V, Poole-Wilson, PA & Sutton, GC 2000, 'Survival of patients with a new diagnosis of heart failure: a population based study', *Heart*, vol. 83, no. 5, May, pp. 505-510.

Crilly, M, Coch, C, Bruce, M, Clark, H & Williams, D 2007, 'Indices of cardiovascular function derived from peripheral pulse wave analysis using radial applanation tonometry: a measurement repeatability study', *Vasc Med*, vol. 12, no. 3, Aug, pp. 189-197.

Currie, MG, Geller, DM, Cole, BR, Siegel, NR, Fok, KF, Adams, SP, Eubanks, SR, Galluppi, GR & Needleman, P 1984, 'Purification and sequence analysis of bioactive atrial peptides (atriopeptins)', *Science*, vol. 223, no. 4631, Jan 6, pp. 67-69.

Damas, JK, Eiken, HG, Oie, E, Bjerkeli, V, Yndestad, A, Ueland, T, Tonnessen, T, Geiran, OR, Aass, H, Simonsen, S, Christensen, G, Froland, SS, Attramadal, H, Gullestad, L & Aukrust, P 2000, 'Myocardial expression of CC- and CXC-chemokines and their receptors in human end-stage heart failure', *Cardiovasc Res*, vol. 47, no. 4, Sep, pp. 778-787.

Damas, JK, Gullestad, L, Aass, H, Simonsen, S, Fjeld, JG, Wikeby, L, Ueland, T, Eiken, HG, Froland, SS & Aukrust, P 2001, 'Enhanced gene expression of chemokines and their corresponding receptors in mononuclear blood cells in chronic heart failure--modulatory effect of intravenous immunoglobulin', *J Am Coll Cardiol*, vol. 38, no. 1, Jul, pp. 187-193.

Damas, JK, Gullestad, L, Ueland, T, Solum, NO, Simonsen, S, Froland, SS & Aukrust, P 2000, 'CXC-chemokines, a new group of cytokines in congestive heart failure--possible role of platelets and monocytes', *Cardiovasc Res*, vol. 45, no. 2, Jan 14, pp. 428-436.

Dandamudi, S & Chen, HH 2012, 'The ASCEND-HF trial: an acute study of clinical effectiveness of nesiritide and decompensated heart failure', *Expert Rev Cardiovasc Ther*, vol. 10, no. 5, May, pp. 557-563.

Dang, PM, Dewas, C, Gaudry, M, Fay, M, Pedruzzi, E, Gougerot-Pocidalo, MA & El Benna, J 1999, 'Priming of human neutrophil respiratory burst by granulocyte/macrophage

colony-stimulating factor (GM-CSF) involves partial phosphorylation of p47(phox)', *J Biol Chem*, vol. 274, no. 29, Jul 16, pp. 20704-20708.

Dang, PM, Stensballe, A, Boussetta, T, Raad, H, Dewas, C, Kroviarski, Y, Hayem, G, Jensen, ON, Gougerot-Pocidalo, MA & El-Benna, J 2006, 'A specific p47phox -serine phosphorylated by convergent MAPKs mediates neutrophil NADPH oxidase priming at inflammatory sites', *J Clin Invest*, vol. 116, no. 7, Jul, pp. 2033-2043.

Daniels, LB & Maisel, AS 2007, 'Natriuretic peptides', *J Am Coll Cardiol*, vol. 50, no. 25, Dec 18, pp. 2357-2368.

Dao, Q, Krishnaswamy, P, Kazanegra, R, Harrison, A, Amirnovin, R, Lenert, L, Clopton, P, Alberto, J, Hlavin, P & Maisel, AS 2001, 'Utility of B-type natriuretic peptide in the diagnosis of congestive heart failure in an urgent-care setting', *J Am Coll Cardiol*, vol. 37, no. 2, Feb, pp. 379-385.

de Bold, AJ, Borenstein, HB, Veress, AT & Sonnenberg, H 1981, 'A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats', *Life Sci*, vol. 28, no. 1, Jan 5, pp. 89-94.

de Bold, AJ, Bruneau, BG & Kuroski de Bold, ML 1996, 'Mechanical and neuroendocrine regulation of the endocrine heart', *Cardiovasc Res*, vol. 31, no. 1, Jan, pp. 7-18.

de Lemos, JA, Morrow, DA, Bentley, JH, Omland, T, Sabatine, MS, McCabe, CH, Hall, C, Cannon, CP & Braunwald, E 2001, 'The prognostic value of B-type natriuretic peptide in patients with acute coronary syndromes', *N Engl J Med*, vol. 345, no. 14, Oct 4, pp. 1014-1021.

De Vito, P, Incerpi, S, Pedersen, JZ & Luly, P 2010, 'Atrial natriuretic peptide and oxidative stress', *Peptides*, vol. 31, no. 7, Jul, pp. 1412-1419.

DeLeo, FR & Quinn, MT 1996, 'Assembly of the phagocyte NADPH oxidase: molecular interaction of oxidase proteins', *J Leukoc Biol*, vol. 60, no. 6, Dec, pp. 677-691.

DeLeo, FR, Renee, J, McCormick, S, Nakamura, M, Apicella, M, Weiss, JP & Nauseef, WM 1998, 'Neutrophils exposed to bacterial lipopolysaccharide upregulate NADPH oxidase assembly', *J Clin Invest*, vol. 101, no. 2, Jan 15, pp. 455-463.

Deswal, A, Petersen, NJ, Feldman, AM, Young, JB, White, BG & Mann, DL 2001, 'Cytokines and cytokine receptors in advanced heart failure: an analysis of the cytokine

database from the Vesnarinone trial (VEST)', *Circulation*, vol. 103, no. 16, Apr 24, pp. 2055-2059.

Devereux, RB, Liebson, PR & Horan, MJ 1987, 'Recommendations concerning use of echocardiography in hypertension and general population research', *Hypertension*, vol. 9, no. 2 Pt 2, Feb, pp. Ii97-104.

Dewas, C, Dang, PM, Gougerot-Pocidalo, MA & El-Benna, J 2003, 'TNF-alpha induces phosphorylation of p47(phox) in human neutrophils: partial phosphorylation of p47phox is a common event of priming of human neutrophils by TNF-alpha and granulocyte-macrophage colony-stimulating factor', *J Immunol*, vol. 171, no. 8, Oct 15, pp. 4392-4398.

Dickey, DM, Dries, DL, Margulies, KB & Potter, LR 2012, 'Guanylyl cyclase (GC)-A and GC-B activities in ventricles and cardiomyocytes from failed and non-failed human hearts: GC-A is inactive in the failed cardiomyocyte', *J Mol Cell Cardiol*, vol. 52, no. 3, Mar, pp. 727-732.

Dickey, DM, Flora, DR, Bryan, PM, Xu, X, Chen, Y & Potter, LR 2007, 'Differential regulation of membrane guanylyl cyclases in congestive heart failure: natriuretic peptide receptor (NPR)-B, Not NPR-A, is the predominant natriuretic peptide receptor in the failing heart', *Endocrinology*, vol. 148, no. 7, Jul, pp. 3518-3522.

DiDonato, JA, Aulak, K, Huang, Y, Wagner, M, Gerstenecker, G, Topbas, C, Gogonea, V, DiDonato, AJ, Tang, WH, Mehl, RA, Fox, PL, Plow, EF, Smith, JD, Fisher, EA & Hazen, SL 2014, 'Site-specific nitration of apolipoprotein A-I at tyrosine 166 is both abundant within human atherosclerotic plaque and dysfunctional', *J Biol Chem*, vol. 289, no. 15, Apr 11, pp. 10276-10292.

Dikalov, S, Griendling, KK & Harrison, DG 2007, 'Measurement of reactive oxygen species in cardiovascular studies', *Hypertension*, vol. 49, no. 4, Apr, pp. 717-727.

Dikalov, S, Jiang, JJ & Mason, RP 2005, 'Characterization of the high-resolution ESR spectra of superoxide radical adducts of 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide (DEPMPO) and 5,5-dimethyl-1-pyrroline N-oxide (DMPO). Analysis of conformational exchange', *Free Radical Research*, vol. 39, no. 8, Aug, pp. 825-836.

Dikalov, S, Skatchkov, M & Bassenge, E 1997, 'Spin trapping of superoxide radicals and peroxynitrite by 1-hydroxy-3-carboxy-pyrrolidine and 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine and the stability of corresponding nitroxyl radicals towards biological reductants', *Biochemical and Biophysical Research Communications*, vol. 231, no. 3, Feb 24, pp. 701-704.

Dikalov, S, Skatchkov, M, Fink, B & Bassenge, E 1997, 'Quantification of superoxide radicals and peroxynitrite in vascular cells using oxidation of sterically hindered hydroxylamines and electron spin resonance', *Nitric Oxide*, vol. 1, no. 5, Oct, pp. 423-431.

Dikalov, SI, Dikalova, AE & Mason, RP 2002, 'Noninvasive diagnostic tool for inflammation-induced oxidative stress using electron spin resonance spectroscopy and an extracellular cyclic hydroxylamine', *Arch Biochem Biophys*, vol. 402, no. 2, Jun 15, pp. 218-226.

Dikalov, SI, Kirilyuk, IA, Voinov, M & Grigor'ev, IA 2011, 'EPR detection of cellular and mitochondrial superoxide using cyclic hydroxylamines', *Free Radic Res*, vol. 45, no. 4, Apr, pp. 417-430.

Dikalova, AE, Kadiiska, MB & Mason, RP 2001, 'An in vivo ESR spin-trapping study: Free radical generation in rats from formate intoxication - role of the Fenton reaction', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 24, Nov 20, pp. 13549-13553.

Ding, J, Knaus, UG, Lian, JP, Bokoch, GM & Badwey, JA 1996, 'The renaturable 69- and 63-kDa protein kinases that undergo rapid activation in chemoattractant-stimulated guinea pig neutrophils are p21-activated kinases', *J Biol Chem*, vol. 271, no. 40, Oct 4, pp. 24869-24873.

Doerries, C, Grote, K, Hilfiker-Kleiner, D, Luchtefeld, M, Schaefer, A, Holland, SM, Sorrentino, S, Manes, C, Schieffer, B, Drexler, H & Landmesser, U 2007, 'Critical role of the NAD(P)H oxidase subunit p47phox for left ventricular remodeling/dysfunction and survival after myocardial infarction', *Circ Res*, vol. 100, no. 6, Mar 30, pp. 894-903.

Dote, K, Sato, H, Tateishi, H, Uchida, T & Ishihara, M 1991, '[Myocardial stunning due to simultaneous multivessel coronary spasms: a review of 5 cases]', *J Cardiol*, vol. 21, no. 2, pp. 203-214.

Drapier, JC, Wietzerbin, J & Hibbs, JB 1988, 'Interferon-Gamma and Tumor Necrosis Factor Induce the L-Arginine-Dependent Cyto-Toxic Effector Mechanism in Murine Macrophages', *European Journal of Immunology*, vol. 18, no. 10, Oct, pp. 1587-1592.

Dries, DL 2011, 'Process matters: Emerging concepts underlying impaired natriuretic peptide system function in heart failure', *Circ Heart Fail*, vol. 4, no. 2, Mar, pp. 107-110.

Duckelmann, C, Mittermayer, F, Haider, DG, Altenberger, J, Eichinger, J & Wolzt, M 2007, 'Asymmetric dimethylarginine enhances cardiovascular risk prediction in patients

with chronic heart failure', *Arterioscler Thromb Vasc Biol*, vol. 27, no. 9, Sep, pp. 2037-2042.

Dzau, VJ 1988, 'Vascular and renal prostaglandins as counter-regulatory systems in heart failure', *Eur Heart J*, vol. 9 Suppl H, Jun, pp. 15-19.

Dzau, VJ, Packer, M, Lilly, LS, Swartz, SL, Hollenberg, NK & Williams, GH 1984, 'Prostaglandins in severe congestive heart failure. Relation to activation of the renin--angiotensin system and hyponatremia', *N Engl J Med*, vol. 310, no. 6, Feb 9, pp. 347-352.

Eiken, HG, Oie, E, Damas, JK, Yndestad, A, Bjerkeli, V, Aass, H, Simonsen, S, Geiran, OR, Tonnessen, T, Christensen, G, Froland, SS, Gullestad, L, Attramadal, H & Aukrust, P 2001, 'Myocardial gene expression of leukaemia inhibitory factor, interleukin-6 and glycoprotein 130 in end-stage human heart failure', *Eur J Clin Invest*, vol. 31, no. 5, May, pp. 389-397.

Eiserich, JP, Cross, CE, Jones, AD, Halliwell, B & van der Vliet, A 1996, 'Formation of nitrating and chlorinating species by reaction of nitrite with hypochlorous acid. A novel mechanism for nitric oxide-mediated protein modification', *J Biol Chem*, vol. 271, no. 32, Aug 9, pp. 19199-19208.

Eitel, I, Lucke, C, Grothoff, M, Sareban, M, Schuler, G, Thiele, H & Gutberlet, M 2010, 'Inflammation in takotsubo cardiomyopathy: insights from cardiovascular magnetic resonance imaging', *Eur Radiol*, vol. 20, no. 2, Feb, pp. 422-431.

Ekelund, UE, Harrison, RW, Shokek, O, Thakkar, RN, Tunin, RS, Senzaki, H, Kass, DA, Marban, E & Hare, JM 1999, 'Intravenous allopurinol decreases myocardial oxygen consumption and increases mechanical efficiency in dogs with pacing-induced heart failure', *Circ Res*, vol. 85, no. 5, Sep 3, pp. 437-445.

El-Benna, J, Dang, PM & Gougerot-Pocidalo, MA 2008, 'Priming of the neutrophil NADPH oxidase activation: role of p47phox phosphorylation and NOX2 mobilization to the plasma membrane', *Semin Immunopathol*, vol. 30, no. 3, Jul, pp. 279-289.

El-Benna, J, Dang, PM, Gougerot-Pocidalo, MA, Marie, JC & Braut-Boucher, F 2009, 'p47phox, the phagocyte NADPH oxidase/NOX2 organizer: structure, phosphorylation and implication in diseases', *Exp Mol Med*, vol. 41, no. 4, Apr 30, pp. 217-225.

el Benna, J, Faust, LP & Babior, BM 1994, 'The phosphorylation of the respiratory burst oxidase component p47phox during neutrophil activation. Phosphorylation of sites

recognized by protein kinase C and by proline-directed kinases', *J Biol Chem*, vol. 269, no. 38, Sep 23, pp. 23431-23436.

El Benna, J, Faust, RP, Johnson, JL & Babior, BM 1996, 'Phosphorylation of the respiratory burst oxidase subunit p47phox as determined by two-dimensional phosphopeptide mapping. Phosphorylation by protein kinase C, protein kinase A, and a mitogen-activated protein kinase', *J Biol Chem*, vol. 271, no. 11, Mar 15, pp. 6374-6378.

Elesgaray, R, Caniffi, C, Ierace, DR, Jaime, MF, Fellet, A, Arranz, C & Costa, MA 2008, 'Signaling cascade that mediates endothelial nitric oxide synthase activation induced by atrial natriuretic peptide', *Regul Pept*, vol. 151, no. 1-3, Nov 29, pp. 130-134.

Elferink, JG & de Koster, BM 1992, 'N-methyl-N'-nitro-N-nitrosoguanidine: the effect on migration and cGMP level of neutrophils', *Toxicology*, vol. 73, no. 1, pp. 45-52.

Ennezat, PV, Vannesson, C, Bouabdallaoui, N, Marechaux, S, Asseman, P & LeJemtel, TH 2011, 'Imagine how many lives you save: angiotensin-converting enzyme inhibition for atherosclerotic vascular disease in the present era of risk reduction', *Expert Opin Pharmacother*, vol. 12, no. 6, Apr, pp. 883-897.

Ervens, J, Schultz, G & Seifert, R 1991, 'Differential inhibition and potentiation of chemoattractant-induced superoxide formation in human neutrophils by the cell-permeant analogue of cyclic GMP, N2,2'-O-dibutyryl guanosine 3':5'-cyclic monophosphate', *Naunyn Schmiedebergs Arch Pharmacol*, vol. 343, no. 4, Apr, pp. 370-376.

Faust, LR, el Benna, J, Babior, BM & Chanock, SJ 1995, 'The phosphorylation targets of p47phox, a subunit of the respiratory burst oxidase. Functions of the individual target serines as evaluated by site-directed mutagenesis', *J Clin Invest*, vol. 96, no. 3, Sep, pp. 1499-1505.

Ferrara, R, Mastrorilli, F, Pasanisi, G, Censi, S, D'Aiello, N, Fucili, A, Valgimigli, M & Ferrari, R 2002, 'Neurohormonal modulation in chronic heart failure', *European Heart Journal Supplements*, vol. 4, no. D, Apr, pp. D3-D11.

Figueroa, MS & Peters, JI 2006, 'Congestive heart failure: Diagnosis, pathophysiology, therapy, and implications for respiratory care', *Respir Care*, vol. 51, no. 4, Apr, pp. 403-412.

Fink, B, Dikalov, S & Bassenge, E 2000, 'A new approach for extracellular spin trapping of nitroglycerin-induced superoxide radicals both in vitro and in vivo', *Free Radic Biol Med*, vol. 28, no. 1, Jan 1, pp. 121-128.

Finley, JJt, Konstam, MA & Udelson, JE 2008, 'Arginine vasopressin antagonists for the treatment of heart failure and hyponatremia', *Circulation*, vol. 118, no. 4, Jul 22, pp. 410-421.

Fischer, D, Rossa, S, Landmesser, U, Spiekermann, S, Engberding, N, Hornig, B & Drexler, H 2005, 'Endothelial dysfunction in patients with chronic heart failure is independently associated with increased incidence of hospitalization, cardiac transplantation, or death', *Eur Heart J*, vol. 26, no. 1, Jan, pp. 65-69.

Flora, DR & Potter, LR 2010, 'Prolonged atrial natriuretic peptide exposure stimulates guanylyl cyclase-a degradation', *Endocrinology*, vol. 151, no. 6, Jun, pp. 2769-2776.

Flynn, TG, de Bold, ML & de Bold, AJ 1983, 'The amino acid sequence of an atrial peptide with potent diuretic and natriuretic properties', *Biochem Biophys Res Commun*, vol. 117, no. 3, Dec 28, pp. 859-865.

Folkert, VW & Schlondorff, D 1979, 'Prostaglandin synthesis in isolated glomeruli', *Prostaglandins*, vol. 17, no. 1, Jan, pp. 79-86.

Forfia, PR, Lee, M, Tunin, RS, Mahmud, M, Champion, HC & Kass, DA 2007, 'Acute phosphodiesterase 5 inhibition mimics hemodynamic effects of B-type natriuretic peptide and potentiates B-type natriuretic peptide effects in failing but not normal canine heart', *J Am Coll Cardiol*, vol. 49, no. 10, Mar 13, pp. 1079-1088.

Forstermann, U, Schmidt, HH, Pollock, JS, Sheng, H, Mitchell, JA, Warner, TD, Nakane, M & Murad, F 1991, 'Isoforms of nitric oxide synthase. Characterization and purification from different cell types', *Biochem Pharmacol*, vol. 42, no. 10, Oct 24, pp. 1849-1857.

Francis, GS, Benedict, C, Johnstone, DE, Kirlin, PC, Nicklas, J, Liang, CS, Kubo, SH, Rudin-Toretsky, E & Yusuf, S 1990, 'Comparison of neuroendocrine activation in patients with left ventricular dysfunction with and without congestive heart failure. A substudy of the Studies of Left Ventricular Dysfunction (SOLVD)', *Circulation*, vol. 82, no. 5, Nov, pp. 1724-1729.

Francis, GS, Goldsmith, SR, Levine, TB, Olivari, MT & Cohn, JN 1984, 'The neurohumoral axis in congestive heart failure', *Ann Intern Med*, vol. 101, no. 3, Sep, pp. 370-377.

Francis, GS, Goldsmith, SR, Ziesche, SM & Cohn, JN 1982, 'Response of plasma norepinephrine and epinephrine to dynamic exercise in patients with congestive heart failure', *Am J Cardiol*, vol. 49, no. 5, Apr 1, pp. 1152-1156.

Francis, SH, Turko, IV & Corbin, JD 2001, 'Cyclic nucleotide phosphodiesterases: relating structure and function', *Prog Nucleic Acid Res Mol Biol*, vol. 65, pp. 1-52.

Frejaville, C, Karoui, H, Tuccio, B, Lemoigne, F, Culcasi, M, Pietri, S, Lauricella, R & Tordo, P 1995, '5-(Diethoxyphosphoryl)-5-Methyl-1-Pyrroline N-Oxide - a New Efficient Phosphorylated Nitrone for the in-Vitro and in-Vivo Spin-Trapping of Oxygen-Centered Radicals', *Journal of Medicinal Chemistry*, vol. 38, no. 2, Jan 20, pp. 258-265.

Friedl, W, Mair, J, Thomas, S, Pichler, M & Puschendorf, B 1999, 'Relationship between natriuretic peptides and hemodynamics in patients with heart failure at rest and after ergometric exercise', *Clin Chim Acta*, vol. 281, no. 1-2, Mar, pp. 121-126.

Friedrichs, K, Adam, M, Remane, L, Mollenhauer, M, Rudolph, V, Rudolph, TK, Andrie, RP, Stockigt, F, Schrickel, JW, Ravekes, T, Deuschl, F, Nickenig, G, Willems, S, Baldus, S & Klinke, A 2014, 'Induction of Atrial Fibrillation by Neutrophils Critically Depends on CD11b/CD18 Integrins', *PLoS One*, vol. 9, no. 2, p. e89307.

Fujii, H, Ichimori, K, Hoshiai, K & Nakazawa, H 1997, 'Nitric oxide inactivates NADPH oxidase in pig neutrophils by inhibiting its assembling process', *J Biol Chem*, vol. 272, no. 52, Dec 26, pp. 32773-32778.

Gaertner, R, Lepailleur-Enouf, D, Gonzalez, W, Nicoletti, A, Mandet, C, Philippe, M, Mercadier, JJ & Michel, JB 2003, 'Pulmonary endothelium as a site of synthesis and storage of interleukin-6 in experimental congestive heart failure', *Eur J Heart Fail*, vol. 5, no. 4, Aug, pp. 435-442.

Gaffney, TE & Braunwald, E 1963, 'Importance of the adrenergic nervous system in the support of circulatory function in patients with congestive heart failure', *Am J Med*, vol. 34, Mar, pp. 320-324.

Garbers, DL 1992, 'Guanylyl cyclase receptors and their endocrine, paracrine, and autocrine ligands', *Cell*, vol. 71, no. 1, Oct 2, pp. 1-4.

Garcia, R, Bonhomme, MC & Schiffrin, EL 1992, 'Divergent regulation of atrial natriuretic factor receptors in high-output heart failure', *Am J Physiol*, vol. 263, no. 6 Pt 2, Dec, pp. H1790-1797.

Gardner, RS, Ozalp, F, Murday, AJ, Robb, SD & McDonagh, TA 2003, 'N-terminal pro-brain natriuretic peptide. A new gold standard in predicting mortality in patients with advanced heart failure', *Eur Heart J*, vol. 24, no. 19, Oct, pp. 1735-1743.

Garg, UC & Hassid, A 1989, 'Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells', *J Clin Invest*, vol. 83, no. 5, May, pp. 1774-1777.

Garlichs, CD, Zhang, H, Schmeisser, A & Daniel, WG 1999, 'Priming of superoxide anion in polymorphonuclear neutrophils by brain natriuretic peptide', *Life Sciences*, vol. 65, no. 10, Jul 30, pp. 1027-1033.

Gennari, C, Nami, R, Agnusdei, D & Fischer, JA 1990, 'Improved Cardiac-Performance with Human Calcitonin Gene Related Peptide in Patients with Congestive Heart-Failure', *Cardiovascular Research*, vol. 24, no. 3, Mar, pp. 239-241.

Gianni, M, Dentali, F, Grandi, AM, Sumner, G, Hiralal, R & Lonn, E 2006, 'Apical ballooning syndrome or takotsubo cardiomyopathy: a systematic review', *Eur Heart J*, vol. 27, no. 13, Jul, pp. 1523-1529.

Giordano, FJ 2005, 'Oxygen, oxidative stress, hypoxia, and heart failure', *J Clin Invest*, vol. 115, no. 3, Mar, pp. 500-508.

Go, AS, Mozaffarian, D, Roger, VL, Benjamin, EJ, Berry, JD, Borden, WB, Bravata, DM, Dai, S, Ford, ES, Fox, CS, Franco, S, Fullerton, HJ, Gillespie, C, Hailpern, SM, Heit, JA, Howard, VJ, Huffman, MD, Kissela, BM, Kittner, SJ, Lackland, DT, Lichtman, JH, Lisabeth, LD, Magid, D, Marcus, GM, Marelli, A, Matchar, DB, McGuire, DK, Mohler, ER, Moy, CS, Mussolino, ME, Nichol, G, Paynter, NP, Schreiner, PJ, Sorlie, PD, Stein, J, Turan, TN, Virani, SS, Wong, ND, Woo, D & Turner, MB 2013, 'Executive summary: heart disease and stroke statistics--2013 update: a report from the American Heart Association', *Circulation*, vol. 127, no. 1, Jan 1, pp. 143-152.

Goetze, JP, Christoffersen, C, Perko, M, Arendrup, H, Rehfeld, JF, Kastrup, J & Nielsen, LB 2003, 'Increased cardiac BNP expression associated with myocardial ischemia', *Faseb Journal*, vol. 17, no. 9, Jun, pp. 1105-1107.

Goldmann, BU, Rudolph, V, Rudolph, TK, Holle, AK, Hillebrandt, M, Meinertz, T & Baldus, S 2009, 'Neutrophil activation precedes myocardial injury in patients with acute myocardial infarction', *Free Radic Biol Med*, vol. 47, no. 1, Jul 1, pp. 79-83.

Goldsmith, SR 1987, 'Vasopressin as vasopressor', *Am J Med*, vol. 82, no. 6, Jun, pp. 1213-1219.

Goldsmith, SR, Francis, GS, Cowley, AW, Jr., Goldenberg, IF & Cohn, JN 1986, 'Hemodynamic effects of infused arginine vasopressin in congestive heart failure', *J Am Coll Cardiol*, vol. 8, no. 4, Oct, pp. 779-783.

Goldsmith, SR, Francis, GS, Cowley, AW, Jr., Levine, TB & Cohn, JN 1983, 'Increased plasma arginine vasopressin levels in patients with congestive heart failure', *J Am Coll Cardiol*, vol. 1, no. 6, Jun, pp. 1385-1390.

Goodwin, DC, Landino, LM & Marnett, LJ 1999, 'Effects of nitric oxide and nitric oxide-derived species on prostaglandin endoperoxide synthase and prostaglandin biosynthesis', *Faseb Journal*, vol. 13, no. 10, Jul, pp. 1121-1136.

Gopi, V, Parthasarathy, A, Umadevi, S & Vellaichamy, E 2013, 'Angiotensin-II down-regulates cardiac natriuretic peptide receptor-A mediated anti-hypertrophic signaling in experimental rat hearts', *Indian J Exp Biol*, vol. 51, no. 1, Jan, pp. 48-55.

Gorlach, A, Brandes, RP, Nguyen, K, Amidi, M, Dehghani, F & Busse, R 2000, 'A gp91phox containing NADPH oxidase selectively expressed in endothelial cells is a major source of oxygen radical generation in the arterial wall', *Circ Res*, vol. 87, no. 1, Jul 7, pp. 26-32.

Grabowski, M, Filipiak, KJ, Malek, LA, Piatkowski, R, Scislo, P, Karpinski, G & Opolski, G 2008, 'Increased B-type natriuretic peptide levels in patients with apical ballooning syndrome - consecutive cases report', *Int J Cardiol*, vol. 124, no. 3, Mar 14, pp. 404-406.

Griendling, KK, Minieri, CA, Ollerenshaw, JD & Alexander, RW 1994, 'Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells', *Circ Res*, vol. 74, no. 6, Jun, pp. 1141-1148.

Griendling, KK, Sorescu, D & Ushio-Fukai, M 2000, 'NAD(P)H oxidase: role in cardiovascular biology and disease', *Circ Res*, vol. 86, no. 5, Mar 17, pp. 494-501.

Griendling, KK, Ushio-Fukai, M, Lassegue, B & Alexander, RW 1997, 'Angiotensin II signaling in vascular smooth muscle. New concepts', *Hypertension*, vol. 29, no. 1 Pt 2, Jan, pp. 366-373.

Grosser, N & Schroder, H 2003, 'Aspirin protects endothelial cells from oxidant damage via the nitric oxide-cGMP pathway', *Arterioscler Thromb Vasc Biol*, vol. 23, no. 8, Aug 1, pp. 1345-1351.

Guzik, TJ, Korbut, R & Adamek-Guzik, T 2003, 'Nitric oxide and superoxide in inflammation and immune regulation', *Journal of Physiology and Pharmacology*, vol. 54, no. 4, Dec, pp. 469-487.

Guzik, TJ, West, NE, Black, E, McDonald, D, Ratnatunga, C, Pillai, R & Channon, KM 2000, 'Vascular superoxide production by NAD(P)H oxidase: association with endothelial dysfunction and clinical risk factors', *Circ Res*, vol. 86, no. 9, May 12, pp. E85-90.

Halcox, JP, Schenke, WH, Zalos, G, Mincemoyer, R, Prasad, A, Waclawiw, MA, Nour, KR & Quyyumi, AA 2002, 'Prognostic value of coronary vascular endothelial dysfunction', *Circulation*, vol. 106, no. 6, Aug 6, pp. 653-658.

Haneda, M, Kikkawa, R, Maeda, S, Togawa, M, Koya, D, Horide, N, Kajiwara, N & Shigeta, Y 1991, 'Dual mechanism of angiotensin II inhibits ANP-induced mesangial cGMP accumulation', *Kidney Int*, vol. 40, no. 2, Aug, pp. 188-194.

Hansen, PR 1995, 'Role of neutrophils in myocardial ischemia and reperfusion', *Circulation*, vol. 91, no. 6, Mar 15, pp. 1872-1885.

Hasegawa, K, Fujiwara, H, Doyama, K, Miyamae, M, Fujiwara, T, Suga, S, Mukoyama, M, Nakao, K, Imura, H & Sasayama, S 1993, 'Ventricular expression of brain natriuretic peptide in hypertrophic cardiomyopathy', *Circulation*, vol. 88, no. 2, Aug, pp. 372-380.

Hassid, A, Konieczkowski, M & Dunn, MJ 1979, 'Prostaglandin synthesis in isolated rat kidney glomeruli', *Proc Natl Acad Sci U S A*, vol. 76, no. 3, Mar, pp. 1155-1159.

Hattori, F, Murayama, N, Noshita, T & Oikawa, S 2003, 'Mitochondrial peroxiredoxin-3 protects hippocampal neurons from excitotoxic injury in vivo', *J Neurochem*, vol. 86, no. 4, Aug, pp. 860-868.

Hawkridge, AM, Heublein, DM, Bergen, HR, 3rd, Cataliotti, A, Burnett, JC, Jr. & Muddiman, DC 2005, 'Quantitative mass spectral evidence for the absence of circulating brain natriuretic peptide (BNP-32) in severe human heart failure', *Proc Natl Acad Sci U S A*, vol. 102, no. 48, Nov 29, pp. 17442-17447.

Hayashi, Y, Sawa, Y, Nishimura, M, Ichikawa, H, Kagisaki, K, Ohtake, S & Matsuda, H 2000, 'Clinical evaluation of leukocyte-depleted blood cardioplegia for pediatric open heart operation', *Ann Thorac Surg*, vol. 69, no. 6, Jun, pp. 1914-1919.

Hayward, CS, Kraidly, M, Webb, CM & Collins, P 2002, 'Assessment of endothelial function using peripheral waveform analysis: a clinical application', *J Am Coll Cardiol*, vol. 40, no. 3, Aug 7, pp. 521-528.

Heitzer, T, Baldus, S, von Kodolitsch, Y, Rudolph, V & Meinertz, T 2005, 'Systemic endothelial dysfunction as an early predictor of adverse outcome in heart failure', *Arterioscler Thromb Vasc Biol*, vol. 25, no. 6, Jun, pp. 1174-1179.

Hematian, S, Siegler, MA & Karlin, KD 2012, 'Heme/copper assembly mediated nitrite and nitric oxide interconversion', *J Am Chem Soc*, vol. 134, no. 46, Nov 21, pp. 18912-18915.

Henry, JP, Gauer, OH & Reeves, JL 1956, 'Evidence of the atrial location of receptors influencing urine flow', *Circ Res*, vol. 4, no. 1, Jan, pp. 85-90.

Heresztyn, T, Worthley, MI & Horowitz, JD 2004, 'Determination of l-arginine and NG, NG - and NG, NG' -dimethyl-L-arginine in plasma by liquid chromatography as AccQ-Fluor fluorescent derivatives', *J Chromatogr B Analyt Technol Biomed Life Sci*, vol. 805, no. 2, Jun 15, pp. 325-329.

Heublein, DM, Clavell, AL, Stingo, AJ, Lerman, A, Wold, L & Burnett, JC, Jr. 1992, 'C-type natriuretic peptide immunoreactivity in human breast vascular endothelial cells', *Peptides*, vol. 13, no. 5, Sep-Oct, pp. 1017-1019.

Heymes, C, Bendall, JK, Ratajczak, P, Cave, AC, Samuel, JL, Hasenfuss, G & Shah, AM 2003, 'Increased myocardial NADPH oxidase activity in human heart failure', *J Am Coll Cardiol*, vol. 41, no. 12, Jun 18, pp. 2164-2171.

Hibbs, JB, Taintor, RR, Vavrin, Z & Rachlin, EM 1988, 'Nitric-Oxide - a Cyto-Toxic Activated Macrophage Effector Molecule', *Biochemical and Biophysical Research Communications*, vol. 157, no. 1, Nov 30, pp. 87-94.

Hill, MF & Singal, PK 1996, 'Antioxidant and oxidative stress changes during heart failure subsequent to myocardial infarction in rats', *Am J Pathol*, vol. 148, no. 1, Jan, pp. 291-300.

Ho, KK, Anderson, KM, Kannel, WB, Grossman, W & Levy, D 1993, 'Survival after the onset of congestive heart failure in Framingham Heart Study subjects', *Circulation*, vol. 88, no. 1, Jul, pp. 107-115.

Ho, KK, Pinsky, JL, Kannel, WB & Levy, D 1993, 'The epidemiology of heart failure: the Framingham Study', *J Am Coll Cardiol*, vol. 22, no. 4 Suppl A, Oct, pp. 6A-13A.

Hobbs, FD, Davis, RC, Roalfe, AK, Hare, R, Davies, MK & Kenkre, JE 2002, 'Reliability of N-terminal pro-brain natriuretic peptide assay in diagnosis of heart failure: cohort study in representative and high risk community populations', *BMJ*, vol. 324, no. 7352, Jun 22, p. 1498.

Hobbs, RE, Miller, LW, Bott-Silverman, C, James, KB, Rincon, G & Grossbard, EB 1996, 'Hemodynamic effects of a single intravenous injection of synthetic human brain natriuretic peptide in patients with heart failure secondary to ischemic or idiopathic dilated cardiomyopathy', *Am J Cardiol*, vol. 78, no. 8, Oct 15, pp. 896-901.

Holmes, SJ, Espiner, EA, Richards, AM, Yandle, TG & Frampton, C 1993, 'Renal, endocrine, and hemodynamic effects of human brain natriuretic peptide in normal man', *J Clin Endocrinol Metab*, vol. 76, no. 1, Jan, pp. 91-96.

Holmgren, A 1995, 'Thioredoxin structure and mechanism: conformational changes on oxidation of the active-site sulfhydryls to a disulfide', *Structure*, vol. 3, no. 3, Mar 15, pp. 239-243.

Horiuchi, M, Akishita, M & Dzau, VJ 1999, 'Recent progress in angiotensin II type 2 receptor research in the cardiovascular system', *Hypertension*, vol. 33, no. 2, Feb, pp. 613-621.

Houng, AK, McNamee, RA, Kerner, A, Sharma, P, Mohamad, A, Tronolone, J & Reed, GL 2009, 'Atrial natriuretic peptide increases inflammation, infarct size, and mortality after experimental coronary occlusion', *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 296, no. 3, Mar, pp. H655-H661.

Hu, G, Huang, X, Zhang, K, Jiang, H & Hu, X 2014, 'Anti-inflammatory Effect of B-Type Natriuretic Peptide Postconditioning During Myocardial Ischemia-Reperfusion: Involvement of PI3K/Akt Signaling Pathway', *Inflammation*, Apr 27.

Huntley, BK, Sandberg, SM, Noser, JA, Cataliotti, A, Redfield, MM, Matsuda, Y & Burnett, JC, Jr. 2006, 'BNP-induced activation of cGMP in human cardiac fibroblasts:

interactions with fibronectin and natriuretic peptide receptors', *J Cell Physiol*, vol. 209, no. 3, Dec, pp. 943-949.

Ichiki, T, Huntley, BK & Burnett, JC, Jr. 2013, 'BNP molecular forms and processing by the cardiac serine protease corin', *Adv Clin Chem*, vol. 61, pp. 1-31.

Ide, T, Tsutsui, H, Kinugawa, S, Utsumi, H, Kang, DC, Hattori, N, Uchida, K, Arimura, K, Egashira, K & Takeshita, A 1999, 'Mitochondrial electron transport complex I is a potential source of oxygen free radicals in the failing myocardium', *Circulation Research*, vol. 85, no. 4, Aug 20, pp. 357-363.

Inanami, O, Johnson, JL, McAdara, JK, Benna, JE, Faust, LR, Newburger, PE & Babior, BM 1998, 'Activation of the leukocyte NADPH oxidase by phorbol ester requires the phosphorylation of p47PHOX on serine 303 or 304', *J Biol Chem*, vol. 273, no. 16, Apr 17, pp. 9539-9543.

Jacobson, GM, Dourron, HM, Liu, J, Carretero, OA, Reddy, DJ, Andrzejewski, T & Pagano, PJ 2003, 'Novel NAD(P)H oxidase inhibitor suppresses angioplasty-induced superoxide and neointimal hyperplasia of rat carotid artery', *Circ Res*, vol. 92, no. 6, Apr 4, pp. 637-643.

Jaiswal, RK 1992, 'Endothelin inhibits the atrial natriuretic factor stimulated cGMP production by activating the protein kinase C in rat aortic smooth muscle cells', *Biochem Biophys Res Commun*, vol. 182, no. 1, Jan 15, pp. 395-402.

Jamieson, JD & Palade, GE 1964, 'Specific Granules in Atrial Muscle Cells', *J Cell Biol*, vol. 23, Oct, pp. 151-172.

Janabi, M, Yamashita, S, Hirano, K, Sakai, N, Hiraoka, H, Matsumoto, K, Zhang, Z, Nozaki, S & Matsuzawa, Y 2000, 'Oxidized LDL-induced NF-kappa B activation and subsequent expression of proinflammatory genes are defective in monocyte-derived macrophages from CD36-deficient patients', *Arterioscler Thromb Vasc Biol*, vol. 20, no. 8, Aug, pp. 1953-1960.

Janzen, EG 1984, 'Spin Trapping', *Methods in Enzymology*, vol. 105, pp. 188-198.

Johnson, JL, Park, JW, Benna, JE, Faust, LP, Inanami, O & Babior, BM 1998, 'Activation of p47(PHOX), a cytosolic subunit of the leukocyte NADPH oxidase. Phosphorylation of ser-359 or ser-370 precedes phosphorylation at other sites and is required for activity', *J Biol Chem*, vol. 273, no. 52, Dec 25, pp. 35147-35152.

Kalra, PR, Anker, SD, Struthers, AD & Coats, AJ 2001, 'The role of C-type natriuretic peptide in cardiovascular medicine', *Eur Heart J*, vol. 22, no. 12, Jun, pp. 997-1007.

Kang, SW, Chae, HZ, Seo, MS, Kim, K, Baines, IC & Rhee, SG 1998, 'Mammalian peroxiredoxin isoforms can reduce hydrogen peroxide generated in response to growth factors and tumor necrosis factor-alpha', *J Biol Chem*, vol. 273, no. 11, Mar 13, pp. 6297-6302.

Kangawa, K, Fukuda, A, Kubota, I, Hayashi, Y, Minamitake, Y & Matsuo, H 1984, 'Human atrial natriuretic polypeptides (hANP): purification, structure synthesis and biological activity', *J Hypertens Suppl*, vol. 2, no. 3, Dec, pp. S321-323.

Kangawa, K, Fukuda, A, Minamino, N & Matsuo, H 1984, 'Purification and complete amino acid sequence of beta-rat atrial natriuretic polypeptide (beta-rANP) of 5,000 daltons', *Biochem Biophys Res Commun*, vol. 119, no. 3, Mar 30, pp. 933-940.

Kangawa, K & Matsuo, H 1984, 'Purification and complete amino acid sequence of alpha-human atrial natriuretic polypeptide (alpha-hANP)', *Biochem Biophys Res Commun*, vol. 118, no. 1, Jan 13, pp. 131-139.

Kasprzak, JD, Klosinska, M & Drozdz, J 2006, 'Clinical aspects of assessment of endothelial function', *Pharmacol Rep*, vol. 58 Suppl, pp. 33-40.

Katz, SD, Hryniewicz, K, Hriljac, I, Balidemaj, K, Dimayuga, C, Hudaihed, A & Yasskiy, A 2005, 'Vascular endothelial dysfunction and mortality risk in patients with chronic heart failure', *Circulation*, vol. 111, no. 3, Jan 25, pp. 310-314.

Katz, SD, Khan, T, Zeballos, GA, Mathew, L, Potharlanka, P, Knecht, M & Whelan, J 1999, 'Decreased activity of the L-arginine-nitric oxide metabolic pathway in patients with congestive heart failure', *Circulation*, vol. 99, no. 16, Apr 27, pp. 2113-2117.

Kawakami, R, Saito, Y, Kishimoto, I, Harada, M, Kuwahara, K, Takahashi, N, Nakagawa, Y, Nakanishi, M, Tanimoto, K, Usami, S, Yasuno, S, Kinoshita, H, Chusho, H, Tamura, N, Ogawa, Y & Nakao, K 2004, 'Overexpression of brain natriuretic peptide facilitates neutrophil infiltration and cardiac matrix metalloproteinase-9 expression after acute myocardial infarction', *Circulation*, vol. 110, no. 21, Nov 23, pp. 3306-3312.

Kellett, J 2006, 'Prediction of mortality of patients with suspected heart failure by brain natriuretic peptide concentrations > 100 pg/ml: comparison of a clinical model with brain natriuretic peptide concentrations', *Heart*, vol. 92, no. 10, Oct, pp. 1512-1513.

Kiemer, AK, Weber, NC, Furst, R, Bildner, N, Kulhanek-Heinze, S & Vollmar, AM 2002, 'Inhibition of p38 MAPK activation via induction of MKP-1: atrial natriuretic peptide reduces TNF-alpha-induced actin polymerization and endothelial permeability', *Circ Res*, vol. 90, no. 8, May 3, pp. 874-881.

Kimura, T, Iwase, M, Kondo, G, Watanabe, H, Ohashi, M, Ito, D & Nagumo, M 2003, 'Suppressive effect of selective cyclooxygenase-2 inhibitor on cytokine release in human neutrophils', *Int Immunopharmacol*, vol. 3, no. 10-11, Oct, pp. 1519-1528.

Kinlough-Rathbone, RL, Packham, MA & Mustard, JF 1983, 'Platelet aggregation', in LA Harker & TS Zimmerman (eds), *Measurements of Platelet Function*, New York, NY: Churchill Livingstone, pp. 64-91.

Kisch, B 1956, 'The sarcosomes of the heart', *J Biophys Biochem Cytol*, vol. 2, no. 4 Suppl, Jul 25, pp. 361-362.

Kita, T, Kida, O, Kato, J, Nakamura, S, Eto, T, Minamino, N, Kangawa, K, Matsuo, H & Tanaka, K 1989, 'Natriuretic and hypotensive effects of brain natriuretic peptide (BNP) in spontaneously hypertensive rats', *Life Sci*, vol. 44, no. 21, pp. 1541-1545.

Klebanoff, SJ 1980, 'Oxygen metabolism and the toxic properties of phagocytes', *Ann Intern Med*, vol. 93, no. 3, Sep, pp. 480-489.

Klebanoff, SJ 1991, *Myeloperoxidase: occurrence and biological function.*, Boca Raton: CRC Press edn, Peroxidases in Chemistry and Biology.

Koba, S, Gao, Z & Sinoway, LI 2009, 'Oxidative stress and the muscle reflex in heart failure', *J Physiol*, vol. 587, no. Pt 21, Nov 1, pp. 5227-5237.

Koppenol, WH, van Buuren, KJ, Butler, J & Braams, R 1976, 'The kinetics of the reduction of cytochrome c by the superoxide anion radical', *Biochim Biophys Acta*, vol. 449, no. 2, Nov 9, pp. 157-168.

Kotlo, KU, Rasenick, MM & Danziger, RS 2010, 'Evidence for cross-talk between atrial natriuretic peptide and nitric oxide receptors', *Mol Cell Biochem*, vol. 338, no. 1-2, May, pp. 183-189.

Krishnaswamy, P, Lubien, E, Clopton, P, Koon, J, Kazanegra, R, Wanner, E, Gardetto, N, Garcia, A, DeMaria, A & Maisel, AS 2001, 'Utility of B-natriuretic peptide levels in identifying patients with left ventricular systolic or diastolic dysfunction', *Am J Med*, vol. 111, no. 4, Sep, pp. 274-279.

Kuroda, J, Ago, T, Matsushima, S, Zhai, P, Schneider, MD & Sadoshima, J 2010, 'NADPH oxidase 4 (Nox4) is a major source of oxidative stress in the failing heart', *Proc Natl Acad Sci U S A*, vol. 107, no. 35, Aug 31, pp. 15565-15570.

Ladich, E, Otsuka, F & Virmani, R 2014, 'A pathologic study of explanted parachute devices from seven heart failure patients following percutaneous ventricular restoration', *Catheter Cardiovasc Interv*, vol. 83, no. 4, Mar 1, pp. 619-630.

Lainchbury, JG, Burnett, JC, Jr., Meyer, D & Redfield, MM 2000, 'Effects of natriuretic peptides on load and myocardial function in normal and heart failure dogs', *Am J Physiol Heart Circ Physiol*, vol. 278, no. 1, Jan, pp. H33-40.

Lainchbury, JG, Espiner, EA, Frampton, CM, Richards, AM, Yandle, TG & Nicholls, MG 1997, 'Cardiac natriuretic peptides as predictors of mortality', *J Intern Med*, vol. 241, no. 4, Apr, pp. 257-259.

Lainchbury, JG, Espiner, EA, Nicholls, MG & Richards, AM 1997, 'Cardiac hormones: diagnostic and therapeutic potential', *N Z Med J*, vol. 110, no. 1046, Jun 27, pp. 219-221.

Lambeth, JD 2004, 'NOX enzymes and the biology of reactive oxygen', *Nat Rev Immunol*, vol. 4, no. 3, Mar, pp. 181-189.

Landmesser, U, Dikalov, S, Price, SR, McCann, L, Fukai, T, Holland, SM, Mitch, WE & Harrison, DG 2003, 'Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension', *J Clin Invest*, vol. 111, no. 8, Apr, pp. 1201-1209.

Lang, CC, Coutie, WJ, Khong, TK, Choy, AM & Struthers, AD 1991, 'Dietary sodium loading increases plasma brain natriuretic peptide levels in man', *Journal of Hypertension*, vol. 9, no. 9, Sep, pp. 779-782.

Laskowski, A, Woodman, OL, Cao, AH, Drummond, GR, Marshall, T, Kaye, DM & Ritchie, RH 2006, 'Antioxidant actions contribute to the antihypertrophic effects of atrial natriuretic peptide in neonatal rat cardiomyocytes', *Cardiovasc Res*, vol. 72, no. 1, Oct 1, pp. 112-123.

Lassegue, B, Sorescu, D, Szocs, K, Yin, Q, Akers, M, Zhang, Y, Grant, SL, Lambeth, JD & Griendling, KK 2001, 'Novel gp91(phox) homologues in vascular smooth muscle cells : nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways', *Circ Res*, vol. 88, no. 9, May 11, pp. 888-894.

Lau, KL, Kong, SK, Ko, WH, Kwan, HY, Huang, Y & Yao, X 2003, 'cGMP stimulates endoplasmic reticulum Ca(2+)-ATPase in vascular endothelial cells', *Life Sci*, vol. 73, no. 16, Sep 5, pp. 2019-2028.

Le, CT, Hollaar, L, van der Valk, EJ & van der Laarse, A 1993, 'Buthionine sulfoximine reduces the protective capacity of myocytes to withstand peroxide-derived free radical attack', *J Mol Cell Cardiol*, vol. 25, no. 5, May, pp. 519-528.

Lee, CR, Watkins, ML, Patterson, JH, Gattis, W, O'Connor C, M, Gheorghiade, M & Adams, KF, Jr. 2003, 'Vasopressin: a new target for the treatment of heart failure', *Am Heart J*, vol. 146, no. 1, Jul, pp. 9-18.

Leeuwenburgh, C, Hardy, MM, Hazen, SL, Wagner, P, Oh-ishi, S, Steinbrecher, UP & Heinecke, JW 1997, 'Reactive nitrogen intermediates promote low density lipoprotein oxidation in human atherosclerotic intima', *J Biol Chem*, vol. 272, no. 3, Jan 17, pp. 1433-1436.

Leitman, DC, Agnost, VL, Tuan, JJ, Andresen, JW & Murad, F 1987, 'Atrial natriuretic factor and sodium nitroprusside increase cyclic GMP in cultured rat lung fibroblasts by activating different forms of guanylate cyclase', *Biochem J*, vol. 244, no. 1, May 15, pp. 69-74.

Levine, TB, Francis, GS, Goldsmith, SR, Simon, AB & Cohn, JN 1982, 'Activity of the sympathetic nervous system and renin-angiotensin system assessed by plasma hormone levels and their relation to hemodynamic abnormalities in congestive heart failure', *Am J Cardiol*, vol. 49, no. 7, May, pp. 1659-1666.

Li, JP, Levick, SP, DiPette, DJ, Janicki, JS & Supowit, SC 2013, 'Alpha-calcitonin gene-related peptide is protective against pressure overload-induced heart failure', *Regulatory Peptides*, vol. 185, Aug 10, pp. 20-28.

Li, N & Karin, M 1999, 'Is NF-kappaB the sensor of oxidative stress?', *Faseb Journal*, vol. 13, no. 10, Jul, pp. 1137-1143.

Li, YB, Huang, TT, Carlson, EJ, Melov, S, Ursell, PC, Olson, TL, Noble, LJ, Yoshimura, MP, Berger, C, Chan, PH, Wallace, DC & Epstein, CJ 1995, 'Dilated Cardiomyopathy and Neonatal Lethality in Mutant Mice Lacking Manganese Superoxide-Dismutase', *Nature Genetics*, vol. 11, no. 4, Dec, pp. 376-381.

Liang, F, O'Rear, J, Schellenberger, U, Tai, L, Lasecki, M, Schreiner, GF, Apple, FS, Maisel, AS, Pollitt, NS & Protter, AA 2007, 'Evidence for functional heterogeneity of circulating B-type natriuretic peptide', *J Am Coll Cardiol*, vol. 49, no. 10, Mar 13, pp. 1071-1078.

Lijnen, P, van Pelt, J & Fagard, R 2011, 'Modulation of reactive oxygen species and collagen synthesis by Angiotensin II in cardiac fibroblasts', *Febs Journal*, vol. 278, Jun, pp. 286-286.

Lin, EQ, Irvine, JC, Cao, AH, Alexander, AE, Love, JE, Patel, R, McMullen, JR, Kaye, DM, Kemp-Harper, BK & Ritchie, RH 2012, 'Nitroxyl (HNO) Stimulates Soluble Guanylyl Cyclase to Suppress Cardiomyocyte Hypertrophy and Superoxide Generation', *PLoS One*, vol. 7, no. 4, p. e34892.

Lincoln, TM & Cornwell, TL 1993, 'Intracellular cyclic GMP receptor proteins', *Faseb Journal*, vol. 7, no. 2, Feb 1, pp. 328-338.

Litt, MR, Jeremy, RW, Weisman, HF, Winkelstein, JA & Becker, LC 1989, 'Neutrophil depletion limited to reperfusion reduces myocardial infarct size after 90 minutes of ischemia. Evidence for neutrophil-mediated reperfusion injury', *Circulation*, vol. 80, no. 6, Dec, pp. 1816-1827.

Liu, FC, Day, YJ, Liou, JT, Yu, HP & Liao, HR 2012, 'Splitomicin inhibits fMLP-induced superoxide anion production in human neutrophils by activate cAMP/PKA signaling inhibition of ERK pathway', *Eur J Pharmacol*, vol. 688, no. 1-3, Aug 5, pp. 68-75.

Liu, J, Yang, F, Yang, XP, Jankowski, M & Pagano, PJ 2003, 'NAD(P)H oxidase mediates angiotensin II-induced vascular macrophage infiltration and medial hypertrophy', *Arterioscler Thromb Vasc Biol*, vol. 23, no. 5, May 1, pp. 776-782.

Liu, S, Ngo, DT, Stewart, S, Horowitz, JD & Chirkov, YY 2014, 'BNP suppression of neutrophil superoxide generation: mechanistic studies in normal subjects', *Clin Exp Pharmacol Physiol*, No. 41 Aug 12, pp 739-743.

Loria, V, Dato, I, Graziani, F & Biasucci, LM 2008, 'Myeloperoxidase: a new biomarker of inflammation in ischemic heart disease and acute coronary syndromes', *Mediators Inflamm*, vol. 2008, p. 135625.

Lucas, KA, Pitari, GM, Kazerounian, S, Ruiz-Stewart, I, Park, J, Schulz, S, Chepenik, KP & Waldman, SA 2000, 'Guanylyl cyclases and signaling by cyclic GMP', *Pharmacol Rev*, vol. 52, no. 3, Sep, pp. 375-414.

Lucchesi, BR 1990, 'Modulation of leukocyte-mediated myocardial reperfusion injury', *Annu Rev Physiol*, vol. 52, pp. 561-576.

Mabuchi, N, Tsutamoto, T, Wada, A, Ohnishi, M, Maeda, K, Hayashi, M & Kinoshita, M 2002, 'Relationship between interleukin-6 production in the lungs and pulmonary vascular resistance in patients with congestive heart failure', *Chest*, vol. 121, no. 4, Apr, pp. 1195-1202.

Madhani, M, Scotland, RS, MacAllister, RJ & Hobbs, AJ 2003, 'Vascular natriuretic peptide receptor-linked particulate guanylate cyclases are modulated by nitric oxide-cyclic GMP signalling', *Br J Pharmacol*, vol. 139, no. 7, Aug, pp. 1289-1296.

Madhavan, M & Prasad, A 2010, 'Proposed Mayo Clinic criteria for the diagnosis of Tako-Tsubo cardiomyopathy and long-term prognosis', *Herz*, vol. 35, no. 4, Jun, pp. 240-243.

Maeda, K, Tsutamoto, T, Wada, A, Hisanaga, T & Kinoshita, M 1998, 'Plasma brain natriuretic peptide as a biochemical marker of high left ventricular end-diastolic pressure in patients with symptomatic left ventricular dysfunction', *Am Heart J*, vol. 135, no. 5 Pt 1, May, pp. 825-832.

Magga, J, Vuolteenaho, O, Tokola, H, Marttila, M & Ruskoaho, H 1997, 'Involvement of transcriptional and posttranscriptional mechanisms in cardiac overload-induced increase of B-type natriuretic peptide gene expression', *Circ Res*, vol. 81, no. 5, Nov, pp. 694-702.

Maisel, A 2002, 'B-type natriuretic peptide levels: diagnostic and prognostic in congestive heart failure: what's next?', *Circulation*, vol. 105, no. 20, May 21, pp. 2328-2331.

Maisel, AS, Krishnaswamy, P, Nowak, RM, McCord, J, Hollander, JE, Duc, P, Omland, T, Storrow, AB, Abraham, WT, Wu, AH, Clopton, P, Steg, PG, Westheim, A, Knudsen, CW, Perez, A, Kazanegra, R, Herrmann, HC, McCullough, PA & Breathing Not Properly Multinational Study, I 2002, 'Rapid measurement of B-type natriuretic peptide in the emergency diagnosis of heart failure', *N Engl J Med*, vol. 347, no. 3, Jul 18, pp. 161-167.

Marcus, LS, Hart, D, Packer, M, Yushak, M, Medina, N, Danziger, RS, Heitjan, DF & Katz, SD 1996, 'Hemodynamic and renal excretory effects of human brain natriuretic peptide infusion in patients with congestive heart failure. A double-blind, placebo-controlled, randomized crossover trial', *Circulation*, vol. 94, no. 12, Dec 15, pp. 3184-3189.

Margulies, KB & Burnett, JC, Jr. 1994, 'Inhibition of cyclic GMP phosphodiesterases augments renal responses to atrial natriuretic factor in congestive heart failure', *J Card Fail*, vol. 1, no. 1, Oct, pp. 71-80.

Mariappan, N, Elks, CM, Fink, B & Francis, J 2009, 'TNF-induced mitochondrial damage: a link between mitochondrial complex I activity and left ventricular dysfunction', *Free Radic Biol Med*, vol. 46, no. 4, Feb 15, pp. 462-470.

Matsumoto, T, Wada, A, Tsutamoto, T, Omura, T, Yokohama, H, Ohnishi, M, Nakae, I, Takahashi, M & Kinoshita, M 1999, 'Vasorelaxing effects of atrial and brain natriuretic peptides on coronary circulation in heart failure', *Am J Physiol*, vol. 276, no. 6 Pt 2, Jun, pp. H1935-1942.

Matsumura, T, Kugiyama, K, Sugiyama, S, Ohgushi, M, Amanaka, K, Suzuki, M & Yasue, H 1996, 'Neutral endopeptidase 24.11 in neutrophils modulates protective effects of natriuretic peptides against neutrophils-induced endothelial cytotoxity', *J Clin Invest*, vol. 97, no. 10, May 15, pp. 2192-2203.

Matsuo, H & Kangawa, K 1984, 'Human and rat atrial natriuretic polypeptides (hANP & rANP) purification, structure and biological activity', *Clinical and Experimental Hypertension Part a-Theory and Practice*, vol. 6, no. 10-11, pp. 1717-1722.

Matsushima, S, Ide, T, Yamato, M, Matsusaka, H, Hattori, F, Ikeuchi, M, Kubota, T, Sunagawa, K, Hasegawa, Y, Kurihara, T, Oikawa, S, Kinugawa, S & Tsutsui, H 2006, 'Overexpression of mitochondrial peroxiredoxin-3 prevents left ventricular remodeling and failure after myocardial infarction in mice', *Circulation*, vol. 113, no. 14, Apr 11, pp. 1779-1786.

Matsushima, S, Kinugawa, S, Ide, T, Matsusaka, H, Inoue, N, Ohta, Y, Yokota, T, Sunagawa, K & Tsutsui, H 2006, 'Overexpression of glutathione peroxidase attenuates myocardial remodeling and preserves diastolic function in diabetic heart', *Am J Physiol Heart Circ Physiol*, vol. 291, no. 5, Nov, pp. H2237-2245.

McLay, JS, Chatterjee, PK, Jardine, AG & Hawksworth, GM 1995, 'Atrial natriuretic factor modulates nitric oxide production: an ANF-C receptor-mediated effect', *Journal of Hypertension*, vol. 13, no. 6, Jun, pp. 625-630.

McMurray, JJ, Adamopoulos, S, Anker, SD, Auricchio, A, Bohm, M, Dickstein, K, Falk, V, Filippatos, G, Fonseca, C, Gomez-Sanchez, MA, Jaarsma, T, Kober, L, Lip, GY, Maggioni, AP, Parkhomenko, A, Pieske, BM, Popescu, BA, Ronnevik, PK, Rutten, FH, Schwitter, J, Seferovic, P, Stepinska, J, Trindade, PT, Voors, AA, Zannad, F, Zeiher, A & Guidelines, ESCCfP 2012, 'ESC Guidelines for the diagnosis and treatment of acute and

chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC', *Eur Heart J*, vol. 33, no. 14, Jul, pp. 1787-1847.

McMurray, JJ, Kjekshus, J, Gullestad, L, Dunselman, P, Hjalmarson, A, Wedel, H, Lindberg, M, Waagstein, F, Grande, P, Hradec, J, Kamensky, G, Korewicki, J, Kuusi, T, Mach, F, Ranjith, N, Wikstrand, J & Group, CS 2009, 'Effects of statin therapy according to plasma high-sensitivity C-reactive protein concentration in the Controlled Rosuvastatin Multinational Trial in Heart Failure (CORONA): a retrospective analysis', *Circulation*, vol. 120, no. 22, Dec 1, pp. 2188-2196.

McMurray, JJ, Packer, M, Desai, AS, Gong, J, Lefkowitz, MP, Rizkala, AR, Rouleau, JL, Shi, VC, Solomon, SD, Swedberg, K, Zile, MR, Investigators, P-H & Committees 2014, 'Angiotensin-neprilysin inhibition versus enalapril in heart failure', *N Engl J Med*, vol. 371, no. 11, Sep 11, pp. 993-1004.

McPhail, LC, Clayton, CC & Snyderman, R 1984, 'The NADPH oxidase of human polymorphonuclear leukocytes. Evidence for regulation by multiple signals', *J Biol Chem*, vol. 259, no. 9, May 10, pp. 5768-5775.

Mehta, J & Mehta, P 1985, 'Prostacyclin and thromboxane A2 production by human cardiac atrial tissues', *Am Heart J*, vol. 109, no. 1, Jan, pp. 1-3.

Mehta, PK & Griendling, KK 2007, 'Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system', *Am J Physiol Cell Physiol*, vol. 292, no. 1, Jan, pp. C82-97.

Metschnikoff, E 1891, 'Lecture on Phagocytosis and Immunity', *Br Med J*, vol. 1, no. 1570, Jan 31, pp. 213-217.

Mills, RM, LeJemtel, TH, Horton, DP, Liang, C, Lang, R, Silver, MA, Lui, C & Chatterjee, K 1999, 'Sustained hemodynamic effects of an infusion of nesiritide (human b-type natriuretic peptide) in heart failure: a randomized, double-blind, placebo-controlled clinical trial. Natrecor Study Group', *J Am Coll Cardiol*, vol. 34, no. 1, Jul, pp. 155-162.

Misono, KS, Grammer, RT, Fukumi, H & Inagami, T 1984, 'Rat atrial natriuretic factor: isolation, structure and biological activities of four major peptides', *Biochem Biophys Res Commun*, vol. 123, no. 2, Sep 17, pp. 444-451.

Moilanen, E, Vuorinen, P, Kankaanranta, H, Metsa-Ketela, T & Vapaatalo, H 1993, 'Inhibition by nitric oxide-donors of human polymorphonuclear leucocyte functions', *Br J Pharmacol*, vol. 109, no. 3, Jul, pp. 852-858.

Molkentin, JD 2003, 'A friend within the heart: natriuretic peptide receptor signaling', *J Clin Invest*, vol. 111, no. 9, May, pp. 1275-1277.

Moncada, S, Palmer, RM & Higgs, EA 1989, 'Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication', *Biochem Pharmacol*, vol. 38, no. 11, Jun 1, pp. 1709-1715.

Moncada, S, Palmer, RM & Higgs, EA 1991, 'Nitric oxide: physiology, pathophysiology, and pharmacology', *Pharmacol Rev*, vol. 43, no. 2, Jun, pp. 109-142.

Moncada, S, Radomski, MW & Palmer, RMJ 1988, 'Endothelium-Derived Relaxing Factor - Identification as Nitric-Oxide and Role in the Control of Vascular Tone and Platelet-Function', *Biochemical Pharmacology*, vol. 37, no. 13, Jul 1, pp. 2495-2501.

Morbidelli, L, Chang, CH, Douglas, JG, Granger, HJ, Ledda, F & Ziche, M 1996, 'Nitric oxide mediates mitogenic effect of VEGF on coronary venular endothelium', *Am J Physiol*, vol. 270, no. 1 Pt 2, Jan, pp. H411-415.

Morel, O, Sauer, F, Imperiale, A, Cimarelli, S, Blondet, C, Jesel, L, Trinh, A, De Poli, F, Ohlmann, P, Constantinesco, A & Bareiss, P 2009, 'Importance of inflammation and neurohumoral activation in Takotsubo cardiomyopathy', *J Card Fail*, vol. 15, no. 3, Apr, pp. 206-213.

Morikawa, M, Inoue, M, Tokumaru, S & Kogo, H 1995, 'Enhancing and Inhibitory Effects of Nitric-Oxide on Superoxide Anion Generation in Human Polymorphonuclear Leukocytes', *British Journal of Pharmacology*, vol. 115, no. 7, Aug, pp. 1302-1306.

Mrakic-Sposta, S, Gussoni, M, Montorsi, M, Porcelli, S & Vezzoli, A 2012, 'Assessment of a standardized ROS production profile in humans by electron paramagnetic resonance', *Oxid Med Cell Longev*, vol. 2012, p. 973927.

Muders, F, Kromer, EP, Griese, DP, Pfeifer, M, Hense, HW, Riegger, GA & Elsner, D 1997, 'Evaluation of plasma natriuretic peptides as markers for left ventricular dysfunction', *Am Heart J*, vol. 134, no. 3, Sep, pp. 442-449.

Mukoyama, M, Nakao, K, Hosoda, K, Suga, S, Saito, Y, Ogawa, Y, Shirakami, G, Jougasaki, M, Obata, K, Yasue, H & et al. 1991, 'Brain natriuretic peptide as a novel

cardiac hormone in humans. Evidence for an exquisite dual natriuretic peptide system, atrial natriuretic peptide and brain natriuretic peptide', *J Clin Invest*, vol. 87, no. 4, Apr, pp. 1402-1412.

Mukoyama, M, Nakao, K, Saito, Y, Ogawa, Y, Hosoda, K, Suga, S, Shirakami, G, Jougasaki, M & Imura, H 1990, 'Increased human brain natriuretic peptide in congestive heart failure', *N Engl J Med*, vol. 323, no. 11, Sep 13, pp. 757-758.

Muller, D, Schulze, C, Baumeister, H, Buck, F & Richter, D 1992, 'Rat insulin-degrading enzyme: cleavage pattern of the natriuretic peptide hormones ANP, BNP, and CNP revealed by HPLC and mass spectrometry', *Biochemistry*, vol. 31, no. 45, Nov 17, pp. 11138-11143.

Munagala, VK, Burnett, JC, Jr. & Redfield, MM 2004, 'The natriuretic peptides in cardiovascular medicine', *Curr Probl Cardiol*, vol. 29, no. 12, Dec, pp. 707-769.

Munagala, VK, Burnett, JC & Redfield, MM 2004, 'The natriuretic peptides in cardiovascular medicine', *Current Problems in Cardiology*, vol. 29, no. 12, Dec, pp. 707-769.

Murad, F 1994, 'The nitric oxide-cyclic GMP signal transduction system for intracellular and intercellular communication', *Recent Prog Horm Res*, vol. 49, pp. 239-248.

Murad, F 2006, 'Shattuck Lecture. Nitric oxide and cyclic GMP in cell signaling and drug development', *N Engl J Med*, vol. 355, no. 19, Nov 9, pp. 2003-2011.

Murray-Rust, J, Leiper, J, McAlister, M, Phelan, J, Tilley, S, Santa Maria, J, Vallance, P & McDonald, N 2001, 'Structural insights into the hydrolysis of cellular nitric oxide synthase inhibitors by dimethylarginine dimethylaminohydrolase', *Nat Struct Biol*, vol. 8, no. 8, Aug, pp. 679-683.

Nagaya, N, Nishikimi, T, Okano, Y, Uematsu, M, Satoh, T, Kyotani, S, Kuribayashi, S, Hamada, S, Kakishita, M, Nakanishi, N, Takamiya, M, Kunieda, T, Matsuo, H & Kangawa, K 1998, 'Plasma brain natriuretic peptide levels increase in proportion to the extent of right ventricular dysfunction in pulmonary hypertension', *J Am Coll Cardiol*, vol. 31, no. 1, Jan, pp. 202-208.

Nakamura, M, Arakawa, N, Yoshida, H, Makita, S, Niinuma, H & Hiramori, K 1998, 'Vasodilatory effects of B-type natriuretic peptide are impaired in patients with chronic heart failure', *Am Heart J*, vol. 135, no. 3, Mar, pp. 414-420.

Nakamura, S, Naruse, M, Naruse, K, Kawana, M, Nishikawa, T, Hosoda, S, Tanaka, I, Yoshimi, T, Yoshihara, I, Inagami, T & et al. 1991, 'Atrial natriuretic peptide and brain natriuretic peptide coexist in the secretory granules of human cardiac myocytes', *Am J Hypertens*, vol. 4, no. 11, Nov, pp. 909-912.

Nakamura, T, Funayama, H, Yoshimura, A, Tsuruya, Y, Saito, M, Kawakami, M & Ishikawa, SE 2006, 'Possible vascular role of increased plasma arginine vasopressin in congestive heart failure', *Int J Cardiol*, vol. 106, no. 2, Jan 13, pp. 191-195.

Nakao, K, Mukoyama, M, Hosoda, K, Suga, S, Ogawa, Y, Saito, Y, Shirakami, G, Arai, H, Jougasaki, M & Imura, H 1991, 'Biosynthesis, secretion, and receptor selectivity of human brain natriuretic peptide', *Can J Physiol Pharmacol*, vol. 69, no. 10, Oct, pp. 1500-1506.

Nef, HM, Mollmann, H, Kostin, S, Troidl, C, Voss, S, Weber, M, Dill, T, Rolf, A, Brandt, R, Hamm, CW & Elsasser, A 2007, 'Tako-Tsubo cardiomyopathy: intraindividual structural analysis in the acute phase and after functional recovery', *Eur Heart J*, vol. 28, no. 20, Oct, pp. 2456-2464.

Nef, HM, Mollmann, H, Troidl, C, Weber, M, Hamm, C & Elsasser, A 2008, 'Tako-Tsubo cardiomyopathy: NT-proBNP as a reliable parameter of a favourable prognosis?', *Int J Cardiol*, vol. 124, no. 2, Feb 29, pp. 237-238.

Neil, C, Nguyen, TH, Kucia, A, Crouch, B, Sverdlov, A, Chirkov, Y, Mahadavan, G, Selvanayagam, J, Dawson, D, Beltrame, J, Zeitz, C, Unger, S, Redpath, T, Frenneaux, M & Horowitz, J 2012, 'Slowly resolving global myocardial inflammation/oedema in Tako-Tsubo cardiomyopathy: evidence from T2-weighted cardiac MRI', *Heart*, vol. 98, no. 17, Sep, pp. 1278-1284.

Neil, CJ, Nguyen, TH, Sverdlov, AL, Chirkov, YY, Chong, CR, Stansborough, J, Beltrame, JF, Kucia, AM, Zeitz, CJ, Frenneaux, MP & Horowitz, JD 2012, 'Can we make sense of takotsubo cardiomyopathy? An update on pathogenesis, diagnosis and natural history', *Expert Rev Cardiovasc Ther*, vol. 10, no. 2, Feb, pp. 215-221.

Nerem, RM 1993, 'Hemodynamics and the vascular endothelium', *J Biomech Eng*, vol. 115, no. 4B, Nov, pp. 510-514.

Nerem, RM, Alexander, RW, Chappell, DC, Medford, RM, Varner, SE & Taylor, WR 1998, 'The study of the influence of flow on vascular endothelial biology', *Am J Med Sci*, vol. 316, no. 3, Sep, pp. 169-175.

Nerem, RM, Harrison, DG, Taylor, WR & Alexander, RW 1993, 'Hemodynamics and vascular endothelial biology', *J Cardiovasc Pharmacol*, vol. 21 Suppl 1, pp. S6-10.

Nguyen, TH, Neil, CJ, Sverdlov, AL, Mahadavan, G, Chirkov, YY, Kucia, AM, Stansborough, J, Beltrame, JF, Selvanayagam, JB, Zeitz, CJ, Struthers, AD, Frenneaux, MP & Horowitz, JD 2011, 'N-terminal pro-brain natriuretic protein levels in takotsubo cardiomyopathy', *Am J Cardiol*, vol. 108, no. 9, Nov 1, pp. 1316-1321.

Nieminen, MS, Brutsaert, D, Dickstein, K, Drexler, H, Follath, F, Harjola, VP, Hochadel, M, Komajda, M, Lassus, J, Lopez-Sendon, JL, Ponikowski, P & Tavazzi, L 2006, 'EuroHeart Failure Survey II (EHFS II): a survey on hospitalized acute heart failure patients: description of population', *Eur Heart J*, vol. 27, no. 22, Nov, pp. 2725-2736.

Nishikimi, T, Maeda, N & Matsuoka, H 2006, 'The role of natriuretic peptides in cardioprotection', *Cardiovasc Res*, vol. 69, no. 2, Feb 1, pp. 318-328.

Noman, A, Ang, DS, Ogston, S, Lang, CC & Struthers, AD 2010, 'Effect of high-dose allopurinol on exercise in patients with chronic stable angina: a randomised, placebo controlled crossover trial', *Lancet*, vol. 375, no. 9732, Jun 19, pp. 2161-2167.

Nussenzveig, DR, Lewicki, JA & Maack, T 1990, 'Cellular mechanisms of the clearance function of type C receptors of atrial natriuretic factor', *J Biol Chem*, vol. 265, no. 34, Dec 5, pp. 20952-20958.

O'Connor, CM, Starling, RC, Hernandez, AF, Armstrong, PW, Dickstein, K, Hasselblad, V, Heizer, GM, Komajda, M, Massie, BM, McMurray, JJ, Nieminen, MS, Reist, CJ, Rouleau, JL, Swedberg, K, Adams, KF, Jr., Anker, SD, Atar, D, Battler, A, Botero, R, Bohidar, NR, Butler, J, Clausell, N, Corbalan, R, Costanzo, MR, Dahlstrom, U, Deckelbaum, LI, Diaz, R, Dunlap, ME, Ezekowitz, JA, Feldman, D, Felker, GM, Fonarow, GC, Gennevois, D, Gottlieb, SS, Hill, JA, Hollander, JE, Howlett, JG, Hudson, MP, Kociol, RD, Krum, H, Laucevicius, A, Levy, WC, Mendez, GF, Metra, M, Mittal, S, Oh, BH, Pereira, NL, Ponikowski, P, Tang, WH, Tanomsup, S, Teerlink, JR, Triposkiadis, F, Troughton, RW, Voors, AA, Whellan, DJ, Zannad, F & Califf, RM 2011, 'Effect of nesiritide in patients with acute decompensated heart failure', *N Engl J Med*, vol. 365, no. 1, Jul 7, pp. 32-43.

Ogawa, T & de Bold, AJ 2012, 'Brain natriuretic Peptide production and secretion in inflammation', *J Transplant*, vol. 2012, p. 962347.

Ogawa, T, Veinot, JP, Kuroski de Bold, ML, Georgalis, T & de Bold, AJ 2008, 'Angiotensin II receptor antagonism reverts the selective cardiac BNP upregulation and secretion observed in myocarditis', *Am J Physiol Heart Circ Physiol*, vol. 294, no. 6, Jun, pp. H2596-2603.

Ogawa, Y, Nakao, K, Mukoyama, M, Hosoda, K, Shirakami, G, Arai, H, Saito, Y, Suga, S, Jougasaki, M & Imura, H 1991, 'Natriuretic peptides as cardiac hormones in normotensive and spontaneously hypertensive rats. The ventricle is a major site of synthesis and secretion of brain natriuretic peptide', *Circ Res*, vol. 69, no. 2, Aug, pp. 491-500.

Ogawa, Y, Nakao, K, Mukoyama, M, Shirakami, G, Itoh, H, Hosoda, K, Saito, Y, Arai, H, Suga, S, Jougasaki, M & et al. 1990, 'Rat brain natriuretic peptide--tissue distribution and molecular form', *Endocrinology*, vol. 126, no. 4, Apr, pp. 2225-2227.

Okada, M, Matsumori, A, Ono, K, Furukawa, Y, Shioi, T, Iwasaki, A, Matsushima, K & Sasayama, S 1998, 'Cyclic stretch upregulates production of interleukin-8 and monocyte chemotactic and activating factor/monocyte chemoattractant protein-1 in human endothelial cells', *Arterioscler Thromb Vasc Biol*, vol. 18, no. 6, Jun, pp. 894-901.

Okumura, K, Yasue, H, Fujii, H, Kugiyama, K, Matsuyama, K, Yoshimura, M, Jougasaki, M, Kikuta, K, Kato, H, Tanaka, H & et al. 1995, 'Effects of brain (B-type) natriuretic peptide on coronary artery diameter and coronary hemodynamic variables in humans: comparison with effects on systemic hemodynamic variables', *J Am Coll Cardiol*, vol. 25, no. 2, Feb, pp. 342-348.

Orus, J, Roig, E, Perez-Villa, F, Pare, C, Azqueta, M, Filella, X, Heras, M & Sanz, G 2000, 'Prognostic value of serum cytokines in patients with congestive heart failure', *J Heart Lung Transplant*, vol. 19, no. 5, May, pp. 419-425.

Palazzuoli, A, Caputo, M, Calabro, A & Nuti, R 2012, 'Clinical impact of BNP and other emerging biomarkers in heart failure evaluation and management', *Minerva Cardioangiol*, vol. 60, no. 2, Apr, pp. 183-194.

Palmer, RMJ, Ashton, DS & Moncada, S 1988, 'Vascular Endothelial-Cells Synthesize Nitric-Oxide from L-Arginine', *Nature*, vol. 333, no. 6174, Jun 16, pp. 664-666.

Palmer, RMJ, Rees, DD, Ashton, DS & Moncada, S 1988, 'L-Arginine Is the Physiological Precursor for the Formation of Nitric-Oxide in Endothelium-Dependent Relaxation', *Biochemical and Biophysical Research Communications*, vol. 153, no. 3, Jun 30, pp. 1251-1256.

Pankow, K, Wang, Y, Gembardt, F, Krause, E, Sun, X, Krause, G, Schultheiss, HP, Siems, WE & Walther, T 2007, 'Successive action of meprin A and neprilysin catabolizes B-type natriuretic peptide', *Circ Res*, vol. 101, no. 9, Oct 26, pp. 875-882.

Papaharalambus, CA & Griendling, KK 2007, 'Basic mechanisms of oxidative stress and reactive oxygen species in cardiovascular injury', *Trends Cardiovasc Med*, vol. 17, no. 2, Feb, pp. 48-54.

Paravicini, TM, Drummond, GR & Sobey, CG 2004, 'Reactive oxygen species in the cerebral circulation: physiological roles and therapeutic implications for hypertension and stroke', *Drugs*, vol. 64, no. 19, pp. 2143-2157.

Park, SM, Prasad, A, Rihal, C, Bell, MR & Oh, JK 2009, 'Left Ventricular Systolic and Diastolic Function in Patients With Apical Ballooning Syndrome Compared With Patients With Acute Anterior ST-Segment Elevation Myocardial Infarction: A Functional Paradox', *Mayo Clinic Proceedings*, vol. 84, no. 6, Jun, pp. 514-521.

Paur, H, Wright, PT, Sikkel, MB, Tranter, MH, Mansfield, C, O'Gara, P, Stuckey, DJ, Nikolaev, VO, Diakonov, I, Pannell, L, Gong, H, Sun, H, Peters, NS, Petrou, M, Zheng, Z, Gorelik, J, Lyon, AR & Harding, SE 2012, 'High levels of circulating epinephrine trigger apical cardiodepression in a beta2-adrenergic receptor/Gi-dependent manner: a new model of Takotsubo cardiomyopathy', *Circulation*, vol. 126, no. 6, Aug 7, pp. 697-706.

Pedram, A, Razandi, M & Levin, ER 2002, 'Deciphering vascular endothelial cell growth factor/vascular permeability factor signaling to vascular permeability. Inhibition by atrial natriuretic peptide', *J Biol Chem*, vol. 277, no. 46, Nov 15, pp. 44385-44398.

Petty, RG & Pearson, JD 1989, 'Endothelium--the axis of vascular health and disease', *J R Coll Physicians Lond*, vol. 23, no. 2, Apr, pp. 92-102.

Piggott, LA, Hassell, KA, Berkova, Z, Morris, AP, Silberbach, M & Rich, TC 2006, 'Natriuretic peptides and nitric oxide stimulate cGMP synthesis in different cellular compartments', *J Gen Physiol*, vol. 128, no. 1, Jul, pp. 3-14.

Porapakkham, P, Porapakkham, P, Zimmet, H, Billah, B & Krum, H 2010, 'B-type natriuretic peptide-guided heart failure therapy: A meta-analysis', *Arch Intern Med*, vol. 170, no. 6, Mar 22, pp. 507-514.

Potter, LR 2011, 'Natriuretic peptide metabolism, clearance and degradation', *FEBS J*, vol. 278, no. 11, Jun, pp. 1808-1817.

Potter, LR, Abbey-Hosch, S & Dickey, DM 2006, 'Natriuretic peptides, their receptors, and cyclic guanosine monophosphate-dependent signaling functions', *Endocrine Reviews*, vol. 27, no. 1, Feb, pp. 47-72.

Potter, LR & Hunter, T 2001, 'Guanylyl cyclase-linked natriuretic peptide receptors: structure and regulation', *J Biol Chem*, vol. 276, no. 9, Mar 2, pp. 6057-6060.

Pou, S, Pou, WS, Bredt, DS, Snyder, SH & Rosen, GM 1992, 'Generation of superoxide by purified brain nitric oxide synthase', *J Biol Chem*, vol. 267, no. 34, Dec 5, pp. 24173-24176.

Prasad, A, Lerman, A & Rihal, CS 2008, 'Apical ballooning syndrome (Tako-Tsubo or stress cardiomyopathy): a mimic of acute myocardial infarction', *Am Heart J*, vol. 155, no. 3, Mar, pp. 408-417.

Ralat, LA, Guo, Q, Ren, M, Funke, T, Dickey, DM, Potter, LR & Tang, WJ 2011, 'Insulin-degrading enzyme modulates the natriuretic peptide-mediated signaling response', *J Biol Chem*, vol. 286, no. 6, Feb 11, pp. 4670-4679.

Raman, B, Singh, K, Zeitz, CJ & Horowitz, JD 2014, 'Takotsubo cardiomyopathy presenting as S-T elevation myocardial infarction: not gone but forgotten?', *Int J Cardiol*, vol. 172, no. 1, Mar 1, pp. e261-262.

Rambaran, C, Jiang, B, Ritter, JM, Shah, A, Kalra, L & Chowienczyk, PJ 2008, 'Assessment of endothelial function: comparison of the pulse wave response to beta 2-adrenoceptor stimulation with flow mediated dilatation', *Br J Clin Pharmacol*, vol. 65, no. 2, Feb, pp. 238-243.

Ren, B, Wu, H, Yin, R, Xu, L, Jing, H, Li, M, Jiang, F & Wang, Z 2010, 'B-Type Natriuretic Peptide Pretreatment Attenuates Heart Ischemia-Reperfusion Injury in Rats', *Transplantation Proceedings*, vol. 42, no. 10, Dec, pp. 4496-4498.

Rey, FE, Cifuentes, ME, Kiarash, A, Quinn, MT & Pagano, PJ 2001, 'Novel competitive inhibitor of NAD(P)H oxidase assembly attenuates vascular O(2)(-) and systolic blood pressure in mice', *Circ Res*, vol. 89, no. 5, Aug 31, pp. 408-414.

Rivero-Vilches, FJ, de Frutos, S, Saura, M, Rodriguez-Puyol, D & Rodriguez-Puyol, M 2003, 'Differential relaxing responses to particulate or soluble guanylyl cyclase activation on endothelial cells: a mechanism dependent on PKG-I alpha activation by NO/cGMP', *Am J Physiol Cell Physiol*, vol. 285, no. 4, Oct, pp. C891-898.

Rochette, L, Tatou, E, Maupoil, V, Zeller, M, Cottin, Y, Jazayeri, S, Brenot, R, Girard, C, David, M & Vergely, C 2011, 'Atrial and vascular oxidative stress in patients with heart failure', *Cell Physiol Biochem*, vol. 27, no. 5, pp. 497-502.

Rodeheffer, RJ 2004, 'Measuring plasma B-type natriuretic peptide in heart failure: good to go in 2004?', *J Am Coll Cardiol*, vol. 44, no. 4, Aug 18, pp. 740-749.

Rojkind, M, Dominguez-Rosales, JA, Nieto, N & Greenwel, P 2002, 'Role of hydrogen peroxide and oxidative stress in healing responses', *Cell Mol Life Sci*, vol. 59, no. 11, Nov, pp. 1872-1891.

Rosen, GM, Finkelstein, E & Rauckman, EJ 1982, 'A method for the detection of superoxide in biological systems', *Arch Biochem Biophys*, vol. 215, no. 2, May, pp. 367-378.

Rouquette, M, Page, S, Bryant, R, Benboubetra, M, Stevens, CR, Blake, DR, Whish, WD, Harrison, R & Tosh, D 1998, 'Xanthine oxidoreductase is asymmetrically localised on the outer surface of human endothelial and endothelial cells in culture', *Febs Letters*, vol. 426, no. 3, Apr 24, pp. 397-401.

Rubanyi, GM 1993, 'The role of endothelium in cardiovascular homeostasis and diseases', *J Cardiovasc Pharmacol*, vol. 22 Suppl 4, pp. S1-14.

Ruffolo, RR, Jr. & Feuerstein, GZ 1998, 'Neurohormonal activation, oxygen free radicals, and apoptosis in the pathogenesis of congestive heart failure', *J Cardiovasc Pharmacol*, vol. 32 Suppl 1, pp. S22-30.

Ruskoaho, H 2003, 'Cardiac hormones as diagnostic tools in heart failure', *Endocrine Reviews*, vol. 24, no. 3, Jun, pp. 341-356.

Sackner-Bernstein, J & Aaronson, KD 2005, 'Nesiritide--not verified', *N Engl J Med*, vol. 353, no. 14, Oct 6, pp. 1525-1527; author reply 1525-1527.

Sackner-Bernstein, JD, Skopicki, HA & Aaronson, KD 2005, 'Risk of worsening renal function with nesiritide in patients with acutely decompensated heart failure', *Circulation*, vol. 111, no. 12, Mar 29, pp. 1487-1491.

Sagols, E & Priymenko, N 2011, 'Oxidative stress in dog with heart failure: the role of dietary Fatty acids and antioxidants', *Vet Med Int*, vol. 2011, p. 180206.

Sakurada, M, Shichiri, M, Imamura, M, Azuma, H & Hirata, Y 2008, 'Nitric oxide upregulates dimethylarginine dimethylaminohydrolase-2 via cyclic GMP induction in endothelial cells', *Hypertension*, vol. 52, no. 5, Nov, pp. 903-909.

Sam, F, Kerstetter, DL, Pimental, DR, Mulukutla, M, Tabaee, A, Bristow, MR, Colucci, WS & Sawyer, DB 2005, 'Increased reactive oxygen species production and functional alterations in antioxidant enzymes in human failing myocardium', *Journal of Cardiac Failure*, vol. 11, no. 6, Aug, pp. 473-480.

Satoh, S, Ohyama, Y & Hayashi, M 1981, 'Effects of renal nerve stimulation, norepinephrine and angiotensin II on renal blood flow in relation to release of PG-like substances in anesthetized dogs', *Arch Int Pharmacodyn Ther*, vol. 254, no. 2, Dec, pp. 304-316.

Sawa, Y, Matsuda, H, Shimazaki, Y, Kaneko, M, Nishimura, M, Amemiya, A, Sakai, K & Nakano, S 1994, 'Evaluation of leukocyte-depleted terminal blood cardioplegic solution in patients undergoing elective and emergency coronary artery bypass grafting', *J Thorac Cardiovasc Surg*, vol. 108, no. 6, Dec, pp. 1125-1131.

Sawada, Y, Suda, M, Yokoyama, H, Kanda, T, Sakamaki, T, Tanaka, S, Nagai, R, Abe, S & Takeuchi, T 1997, 'Stretch-induced hypertrophic growth of cardiocytes and processing of brain-type natriuretic peptide are controlled by proprotein-processing endoprotease furin', *J Biol Chem*, vol. 272, no. 33, Aug 15, pp. 20545-20554.

Schachinger, V, Britten, MB & Zeiher, AM 2000, 'Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease', *Circulation*, vol. 101, no. 16, Apr 25, pp. 1899-1906.

Schirger, JA, Grantham, JA, Kullo, IJ, Jougasaki, M, Wennberg, PW, Chen, HH, Lisy, O, Miller, V, Simari, RD & Burnett, JC, Jr. 2000, 'Vascular actions of brain natriuretic peptide: modulation by atherosclerosis and neutral endopeptidase inhibition', *J Am Coll Cardiol*, vol. 35, no. 3, Mar 1, pp. 796-801.

Schramm, A, Matusik, P, Osmenda, G & Guzik, TJ 2012, 'Targeting NADPH oxidases in vascular pharmacology', *Vascul Pharmacol*, vol. 56, no. 5-6, May-Jun, pp. 216-231.

Schrier, RW & Abraham, WT 1999, 'Hormones and hemodynamics in heart failure', *N Engl J Med*, vol. 341, no. 8, Aug 19, pp. 577-585.

Seddon, M, Looi, YH & Shah, AM 2007, 'Oxidative stress and redox signalling in cardiac hypertrophy and heart failure', *Heart*, vol. 93, no. 8, Aug, pp. 903-907.

Seshiah, PN, Weber, DS, Rocic, P, Valppu, L, Taniyama, Y & Griendling, KK 2002, 'Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators', *Circ Res*, vol. 91, no. 5, Sep 6, pp. 406-413.

Shah, AM, Spurgeon, HA, Sollott, SJ, Talo, A & Lakatta, EG 1994, '8-bromo-cGMP reduces the myofilament response to Ca2+ in intact cardiac myocytes', *Circ Res*, vol. 74, no. 5, May, pp. 970-978.

Shao, B, Pennathur, S & Heinecke, JW 2012, 'Myeloperoxidase targets apolipoprotein A-I, the major high density lipoprotein protein, for site-specific oxidation in human atherosclerotic lesions', *J Biol Chem*, vol. 287, no. 9, Feb 24, pp. 6375-6386.

Shimizu, H, Masuta, K, Aono, K, Asada, H, Sasakura, K, Tamaki, M, Sugita, K & Yamada, K 2002, 'Molecular forms of human brain natriuretic peptide in plasma', *Clin Chim Acta*, vol. 316, no. 1-2, Feb, pp. 129-135.

Shimokawa, H, Flavahan, NA & Vanhoutte, PM 1991, 'Loss of endothelial pertussis toxin-sensitive G protein function in atherosclerotic porcine coronary arteries', *Circulation*, vol. 83, no. 2, Feb, pp. 652-660.

Shioi, T, Matsumori, A, Kihara, Y, Inoko, M, Ono, K, Iwanaga, Y, Yamada, T, Iwasaki, A, Matsushima, K & Sasayama, S 1997, 'Increased expression of interleukin-1 beta and monocyte chemotactic and activating factor/monocyte chemoattractant protein-1 in the hypertrophied and failing heart with pressure overload', *Circ Res*, vol. 81, no. 5, Nov, pp. 664-671.

Shiomi, T, Tsutsui, H, Matsusaka, H, Murakami, K, Hayashidani, S, Ikeuchi, M, Wen, J, Kubota, T, Utsumi, H & Takeshita, A 2004, 'Overexpression of glutathione peroxidase prevents left ventricular remodeling and failure after myocardial infarction in mice', *Circulation*, vol. 109, no. 4, Feb 3, pp. 544-549.

Siems, WG, Capuozzo, E, Verginelli, D, Salerno, C, Crifo, C & Grune, T 1997, 'Inhibition of NADPH oxidase-mediated superoxide radical formation in PMA-stimulated human neutrophils by 4-hydroxynonenal--binding to -SH and -NH2 groups', *Free Radic Res*, vol. 27, no. 4, Oct, pp. 353-358.

Simmons, WW, Balligand, JL, Ungureanulongrois, D, Michel, T, Kelly, RA & Smith, TW 1994, 'Dexamethasone Regulates Inducible Nitric-Oxide Synthase Activity in Cardiac Microvascular Endothelial-Cells by Suppressing Tetrahydrobiopterin Synthesis', *Circulation*, vol. 90, no. 4, Oct, pp. 627-627.

Singh, G, Kuc, RE, Maguire, JJ, Fidock, M & Davenport, AP 2006, 'Novel snake venom ligand dendroaspis natriuretic peptide is selective for natriuretic peptide receptor-A in human heart: downregulation of natriuretic peptide receptor-A in heart failure', *Circ Res*, vol. 99, no. 2, Jul 21, pp. 183-190.

Singh, K, Balligand, JL, Simmons, WW, Kelly, RA & Smith, TW 1994, 'Cytokine-Activated Signal-Transduction Pathways and Induction of Nitric-Oxide Synthase in Cardiac Myocytes and Microvascular Endothelial-Cells', *Circulation*, vol. 90, no. 4, Oct, pp. 192-192.

Smith, SC, Jr., Blair, SN, Bonow, RO, Brass, LM, Cerqueira, MD, Dracup, K, Fuster, V, Gotto, A, Grundy, SM, Miller, NH, Jacobs, A, Jones, D, Krauss, RM, Mosca, L, Ockene, I, Pasternak, RC, Pearson, T, Pfeffer, MA, Starke, RD & Taubert, KA 2001, 'AHA/ACC Scientific Statement: AHA/ACC guidelines for preventing heart attack and death in patients with atherosclerotic cardiovascular disease: 2001 update: A statement for healthcare professionals from the American Heart Association and the American College of Cardiology', *Circulation*, vol. 104, no. 13, Sep 25, pp. 1577-1579.

Sorescu, GP, Song, H, Tressel, SL, Hwang, J, Dikalov, S, Smith, DA, Boyd, NL, Platt, MO, Lassegue, B, Griendling, KK & Jo, H 2004, 'Bone morphogenic protein 4 produced in endothelial cells by oscillatory shear stress induces monocyte adhesion by stimulating reactive oxygen species production from a nox1-based NADPH oxidase', *Circ Res*, vol. 95, no. 8, Oct 15, pp. 773-779.

Stevenson, LW & Perloff, JK 1989, 'The limited reliability of physical signs for estimating hemodynamics in chronic heart failure', *JAMA*, vol. 261, no. 6, Feb 10, pp. 884-888.

Stewart, S, MacIntyre, K, Hole, DJ, Capewell, S & McMurray, JJ 2001, 'More 'malignant' than cancer? Five-year survival following a first admission for heart failure', *Eur J Heart Fail*, vol. 3, no. 3, Jun, pp. 315-322.

Stingo, AJ, Clavell, AL, Heublein, DM, Wei, CM, Pittelkow, MR & Burnett, JC, Jr. 1992, 'Presence of C-type natriuretic peptide in cultured human endothelial cells and plasma', *Am J Physiol*, vol. 263, no. 4 Pt 2, Oct, pp. H1318-1321.

Stoupakis, G & Klapholz, M 2003, 'Natriuretic peptides: biochemistry, physiology, and therapeutic role in heart failure', *Heart Dis*, vol. 5, no. 3, May-Jun, pp. 215-223.

Struthers, AD 1994, 'Ten years of natriuretic peptide research: a new dawn for their diagnostic and therapeutic use?', *BMJ*, vol. 308, no. 6944, Jun 18, pp. 1615-1619.

Su, J, Scholz, PM & Weiss, HR 2005, 'Differential effects of cGMP produced by soluble and particulate guanylyl cyclase on mouse ventricular myocytes', *Exp Biol Med (Maywood)*, vol. 230, no. 4, Apr, pp. 242-250.

Sudoh, T, Kangawa, K, Minamino, N & Matsuo, H 1988, 'A new natriuretic peptide in porcine brain', *Nature*, vol. 332, no. 6159, Mar 3, pp. 78-81.

Sudoh, T, Minamino, N, Kangawa, K & Matsuo, H 1990, 'C-type natriuretic peptide (CNP): a new member of natriuretic peptide family identified in porcine brain', *Biochem Biophys Res Commun*, vol. 168, no. 2, Apr 30, pp. 863-870.

Sun, T, Wang, L & Zhang, Y 2006, 'Prognostic value of B-type natriuretic peptide in patients with acute coronary syndromes', *Arch Med Res*, vol. 37, no. 4, May, pp. 502-505.

Sun, Y, Zhang, J, Lu, L, Chen, SS, Quinn, MT & Weber, KT 2002, 'Aldosterone-induced inflammation in the rat heart : role of oxidative stress', *Am J Pathol*, vol. 161, no. 5, Nov, pp. 1773-1781.

Sun, YG, Deng, TL, Lu, N, Yan, M & Zheng, XX 2010, 'B-type natriuretic peptide protect cardiomyocytes at reperfusion via mitochondrial calcium uniporter', *Biomedicine & Pharmacotherapy*, vol. 64, no. 3, Mar, pp. 170-176.

Supaporn, T, Sandberg, SM, Borgeson, DD, Heublein, DM, Luchner, A, Wei, CM, Dousa, TP & Burnett, JC, Jr. 1996, 'Blunted cGMP response to agonists and enhanced glomerular cyclic 3',5'-nucleotide phosphodiesterase activities in experimental congestive heart failure', *Kidney Int*, vol. 50, no. 5, Nov, pp. 1718-1725.

Szatalowicz, VL, Arnold, PE, Chaimovitz, C, Bichet, D, Berl, T & Schrier, RW 1981, 'Radioimmunoassay of plasma arginine vasopressin in hyponatremic patients with congestive heart failure', *N Engl J Med*, vol. 305, no. 5, Jul 30, pp. 263-266.

Tamura, N, Ogawa, Y, Yasoda, A, Itoh, H, Saito, Y & Nakao, K 1996, 'Two cardiac natriuretic peptide genes (atrial natriuretic peptide and brain natriuretic peptide) are organized in tandem in the mouse and human genomes', *J Mol Cell Cardiol*, vol. 28, no. 8, Aug, pp. 1811-1815.

Tang, WH, Brennan, ML, Philip, K, Tong, W, Mann, S, Van Lente, F & Hazen, SL 2006, 'Plasma myeloperoxidase levels in patients with chronic heart failure', *Am J Cardiol*, vol. 98, no. 6, Sep 15, pp. 796-799.

Tang, WH, Tong, W, Troughton, RW, Martin, MG, Shrestha, K, Borowski, A, Jasper, S, Hazen, SL & Klein, AL 2007, 'Prognostic value and echocardiographic determinants of plasma myeloperoxidase levels in chronic heart failure', *J Am Coll Cardiol*, vol. 49, no. 24, Jun 19, pp. 2364-2370.

Tanigawa, T, Kotake, Y & Reinke, LA 1993, 'Spin-Trapping of Superoxide from Glass Adherent Polymorphonuclear Leukocytes Induced by N-Formylmethionyl-Leucyl-Phenylalanine', *Free Radical Research Communications*, vol. 19, no. 2, pp. 101-110.

Testa, M, Yeh, M, Lee, P, Fanelli, R, Loperfido, F, Berman, JW & LeJemtel, TH 1996, 'Circulating levels of cytokines and their endogenous modulators in patients with mild to severe congestive heart failure due to coronary artery disease or hypertension', *J Am Coll Cardiol*, vol. 28, no. 4, Oct, pp. 964-971.

Thom, SR, Mendiguren, I, Hardy, K, Bolotin, T, Fisher, D, Nebolon, M & Kilpatrick, L 1997, 'Inhibition of human neutrophil beta2-integrin-dependent adherence by hyperbaric O2', *Am J Physiol*, vol. 272, no. 3 Pt 1, Mar, pp. C770-777.

Toll, L, Brandt, SR, Olsen, CM, Judd, AK & Almquist, RG 1991, 'Isolation and characterization of a new atrial peptide-degrading enzyme from bovine kidney', *Biochem Biophys Res Commun*, vol. 175, no. 3, Mar 29, pp. 886-893.

Tonnessen, T, Florholmen, G, Henriksen, UL & Christensen, G 2003, 'Cardiopulmonary alterations in mRNA expression for interleukin-1beta, the interleukin-6 superfamily and CXC-chemokines during development of postischaemic heart failure in the rat', *Clin Physiol Funct Imaging*, vol. 23, no. 5, Sep, pp. 263-268.

Torre-Amione, G, Kapadia, S, Benedict, C, Oral, H, Young, JB & Mann, DL 1996, 'Proinflammatory cytokine levels in patients with depressed left ventricular ejection fraction: a report from the Studies of Left Ventricular Dysfunction (SOLVD)', *J Am Coll Cardiol*, vol. 27, no. 5, Apr, pp. 1201-1206.

Torre-Amione, G, Kapadia, S, Lee, J, Durand, JB, Bies, RD, Young, JB & Mann, DL 1996, 'Tumor necrosis factor-alpha and tumor necrosis factor receptors in the failing human heart', *Circulation*, vol. 93, no. 4, Feb 15, pp. 704-711.

Tousoulis, D, Charakida, M & Stefanadis, C 2005, 'Inflammation and endothelial dysfunction as therapeutic targets in patients with heart failure', *Int J Cardiol*, vol. 100, no. 3, Apr 28, pp. 347-353.

Tsutamoto, T, Kanamori, T, Morigami, N, Sugimoto, Y, Yamaoka, O & Kinoshita, M 1993, 'Possibility of downregulation of atrial natriuretic peptide receptor coupled to guanylate cyclase in peripheral vascular beds of patients with chronic severe heart failure', *Circulation*, vol. 87, no. 1, Jan, pp. 70-75.

Tsutamoto, T, Wada, A, Maeda, K, Hisanaga, T, Fukai, D, Maeda, Y, Ohnishi, M, Mabuchi, N & Kinoshita, M 1997, 'Digitalis increases brain natriuretic peptide in patients with severe congestive heart failure', *Am Heart J*, vol. 134, no. 5 Pt 1, Nov, pp. 910-916.

Tsutamoto, T, Wada, A, Maeda, K, Hisanaga, T, Maeda, Y, Fukai, D, Ohnishi, M, Sugimoto, Y & Kinoshita, M 1997, 'Attenuation of compensation of endogenous cardiac natriuretic peptide system in chronic heart failure: prognostic role of plasma brain natriuretic peptide concentration in patients with chronic symptomatic left ventricular dysfunction', *Circulation*, vol. 96, no. 2, Jul 15, pp. 509-516.

Tsutsui, H, Kinugawa, S & Matsushima, S 2011, 'Oxidative stress and heart failure', *Am J Physiol Heart Circ Physiol*, vol. 301, no. 6, Dec, pp. H2181-2190.

Tziakas, DN, Chalikias, GK & Xatseras, DI 2003, 'Neurohormonal Hypothesis in Heart Failure', *Hellenic J Cardiol*, vol. 44, pp. 195-205.

Ueda, S, Minamino, N, Aburaya, M, Kangawa, K, Matsukura, S & Matsuo, H 1991, 'Distribution and characterization of immunoreactive porcine C-type natriuretic peptide', *Biochem Biophys Res Commun*, vol. 175, no. 3, Mar 29, pp. 759-767.

Ueland, T, Kjekshus, J, Froland, SS, Omland, T, Squire, IB, Gullestad, L, Dickstein, K & Aukrust, P 2005, 'Plasma levels of soluble tumor necrosis factor receptor type I during the acute phase following complicated myocardial infarction predicts survival in high-risk patients', *J Am Coll Cardiol*, vol. 46, no. 11, Dec 6, pp. 2018-2021.

Ukai, T, Cheng, CP, Tachibana, H, Igawa, A, Zhang, ZS, Cheng, HJ & Little, WC 2001, 'Allopurinol enhances the contractile response to dobutamine and exercise in dogs with pacing-induced heart failure', *Circulation*, vol. 103, no. 5, Feb 6, pp. 750-755.

Vaduganathan, M, Greene, SJ, Butler, J, Sabbah, HN, Shantsila, E, Lip, GY & Gheorghiade, M 2013, 'The immunological axis in heart failure: importance of the leukocyte differential', *Heart Fail Rev*, vol. 18, no. 6, Nov, pp. 835-845.

Valen, G, Yan, ZQ & Hansson, GK 2001, 'Nuclear factor kappa-B and the heart', *J Am Coll Cardiol*, vol. 38, no. 2, Aug, pp. 307-314.

van der Vliet, A, Eiserich, JP, Halliwell, B & Cross, CE 1997, 'Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite. A potential additional mechanism of nitric oxide-dependent toxicity', *J Biol Chem*, vol. 272, no. 12, Mar 21, pp. 7617-7625.

van Empel, VP, Bertrand, AT, van Oort, RJ, van der Nagel, R, Engelen, M, van Rijen, HV, Doevendans, PA, Crijns, HJ, Ackerman, SL, Sluiter, W & De Windt, LJ 2006, 'EUK-8, a superoxide dismutase and catalase mimetic, reduces cardiac oxidative stress and ameliorates pressure overload-induced heart failure in the harlequin mouse mutant', *J Am Coll Cardiol*, vol. 48, no. 4, Aug 15, pp. 824-832.

Vane, JR, Anggard, EE & Botting, RM 1990, 'Regulatory functions of the vascular endothelium', *N Engl J Med*, vol. 323, no. 1, Jul 5, pp. 27-36.

Vasquez-Vivar, J, Kalyanaraman, B, Martasek, P, Hogg, N, Masters, BS, Karoui, H, Tordo, P & Pritchard, KA, Jr. 1998, 'Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors', *Proc Natl Acad Sci U S A*, vol. 95, no. 16, Aug 4, pp. 9220-9225.

Venugopal, J 2001, 'Cardiac natriuretic peptides--hope or hype?', *J Clin Pharm Ther*, vol. 26, no. 1, Feb, pp. 15-31.

Vila, V, Sales, VM, Almenar, L, Lazaro, IS, Villa, P & Reganon, E 2008, 'Effect of oral anticoagulant therapy on thrombospondin-1 and von Willebrand factor in patients with stable heart failure', *Thromb Res*, vol. 121, no. 5, pp. 611-615.

Wambach, G & Koch, J 1995, 'BNP plasma levels during acute volume expansion and chronic sodium loading in normal men', *Clin Exp Hypertens*, vol. 17, no. 4, May, pp. 619-629.

Weber, C 2005, 'Platelets and chemokines in atherosclerosis: partners in crime', *Circ Res*, vol. 96, no. 6, Apr 1, pp. 612-616.

Wedel, B & Garbers, D 2001, 'The guanylyl cyclase family at Y2K', *Annu Rev Physiol*, vol. 63, pp. 215-233.

Wei, CM, Aarhus, LL, Miller, VM & Burnett, JC, Jr. 1993, 'Action of C-type natriuretic peptide in isolated canine arteries and veins', *Am J Physiol*, vol. 264, no. 1 Pt 2, Jan, pp. H71-73.

Wei, CM, Heublein, DM, Perrella, MA, Lerman, A, Rodeheffer, RJ, McGregor, CG, Edwards, WD, Schaff, HV & Burnett, JC, Jr. 1993, 'Natriuretic peptide system in human heart failure', *Circulation*, vol. 88, no. 3, Sep, pp. 1004-1009.

Weil, JA, Bolton, J & Wertz, J 1994, *Electron Paramagnetic Resonance: Elementary Theory and Practical Applications.*, John Wiley and Sons, New York, NY.

Weiss, SJ 1989, 'Tissue destruction by neutrophils', *N Engl J Med*, vol. 320, no. 6, Feb 9, pp. 365-376.

Wenzel-Seifert, K, Ervens, J & Seifert, R 1991, 'Differential inhibition and potentiation by cell-permeant analogues of cyclic AMP and cyclic GMP and NO-containing compounds of exocytosis in human neutrophils', *Naunyn Schmiedebergs Arch Pharmacol*, vol. 344, no. 4, Oct, pp. 396-402.

Werner, CG, Godfrey, V, Arnold, RR, Featherstone, GL, Bender, D, Schlossmann, J, Schiemann, M, Hofmann, F & Pryzwansky, KB 2005, 'Neutrophil dysfunction in guanosine 3',5'-cyclic monophosphate-dependent protein kinase I-deficient mice', *J Immunol*, vol. 175, no. 3, Aug 1, pp. 1919-1929.

Wever, RM, van Dam, T, van Rijn, HJ, de Groot, F & Rabelink, TJ 1997, 'Tetrahydrobiopterin regulates superoxide and nitric oxide generation by recombinant endothelial nitric oxide synthase', *Biochem Biophys Res Commun*, vol. 237, no. 2, Aug 18, pp. 340-344.

White, CR, DarleyUsmar, V, Berrington, WR, McAdams, M, Gore, JZ, Thompson, JA, Parks, DA, Tarpey, MM & Freeman, BA 1996, 'Circulating plasma xanthine oxidase contributes to vascular dysfunction in hypercholesterolemic rabbits', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 16, Aug 6, pp. 8745-8749.

Wiedermann, CJ, Niedermuhlbichler, M, Braunsteiner, H & Widermann, CJ 1992, 'Priming of polymorphonuclear neutrophils by atrial natriuretic peptide in vitro', *J Clin Invest*, vol. 89, no. 5, May, pp. 1580-1586.

Wijbenga, JA, Balk, AH, Boomsma, F, Man in 't Veld, AJ & Hall, C 1999, 'Cardiac peptides differ in their response to exercise. Implications for patients with heart failure in clinical practice', *Eur Heart J*, vol. 20, no. 19, Oct, pp. 1424-1428.

Wilcox, JN, Subramanian, RR, Sundell, CL, Tracey, WR, Pollock, JS, Harrison, DG & Marsden, PA 1997, 'Expression of multiple isoforms of nitric oxide synthase in normal and atherosclerotic vessels', *Arterioscler Thromb Vasc Biol*, vol. 17, no. 11, Nov, pp. 2479-2488.

Wilkinson, IB, Hall, IR, MacCallum, H, Mackenzie, IS, McEniery, CM, van der Arend, BJ, Shu, YE, MacKay, LS, Webb, DJ & Cockcroft, JR 2002, 'Pulse-wave analysis: clinical evaluation of a noninvasive, widely applicable method for assessing endothelial function', *Arterioscler Thromb Vasc Biol*, vol. 22, no. 1, Jan, pp. 147-152.

William, M, Hamilton, EJ, Garcia, A, Bundgaard, H, Chia, KK, Figtree, GA & Rasmussen, HH 2008, 'Natriuretic peptides stimulate the cardiac sodium pump via NPR-C-coupled NOS activation', *Am J Physiol Cell Physiol*, vol. 294, no. 4, Apr, pp. C1067-1073.

Wu, B, Jiang, H, Lin, R, Cui, B, Wen, HZ & Lu, ZB 2009, 'Pretreatment with B-type Natriuretic Peptide Protects the Heart From Ischemia-Reperfusion Injury by Inhibiting Myocardial Apoptosis', *Tohoku Journal of Experimental Medicine*, vol. 219, no. 2, Oct, pp. 107-114.

Wu, C, Wu, F, Pan, J, Morser, J & Wu, Q 2003, 'Furin-mediated processing of Pro-C-type natriuretic peptide', *J Biol Chem*, vol. 278, no. 28, Jul 11, pp. 25847-25852.

Wyatt, TA, Lincoln, TM & Pryzwansky, KB 1993, 'Regulation of human neutrophil degranulation by LY-83583 and L-arginine: role of cGMP-dependent protein kinase', *Am J Physiol*, vol. 265, no. 1 Pt 1, Jul, pp. C201-211.

Xia, WJ, Huang, YY, Chen, YL, Chen, SL & He, JG 2011, 'Acute myocardial ischemia directly modulates the expression of brain natriuretic peptide at the transcriptional and translational levels via inflammatory cytokines', *Eur J Pharmacol*, vol. 670, no. 1, Nov 16, pp. 7-12.

Xia, Y, Tsai, AL, Berka, V & Zweier, JL 1998, 'Superoxide generation from endothelial nitric-oxide synthase. A Ca2+/calmodulin-dependent and tetrahydrobiopterin regulatory process', *J Biol Chem*, vol. 273, no. 40, Oct 2, pp. 25804-25808.

Xu, KY, Huso, DL, Dawson, TM, Bredt, DS & Becker, LC 1999, 'Nitric oxide synthase in cardiac sarcoplasmic reticulum', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 2, Jan 19, pp. 657-662.

Yamamoto, M, Yang, G, Hong, C, Liu, J, Holle, E, Yu, X, Wagner, T, Vatner, SF & Sadoshima, J 2003, 'Inhibition of endogenous thioredoxin in the heart increases oxidative stress and cardiac hypertrophy', *J Clin Invest*, vol. 112, no. 9, Nov, pp. 1395-1406.

Yamazaki, H, Senju, Y, Kinoshita, N, Katsukawa, F & Onishi, S 2000, 'Plasma brain natriuretic peptide in athletes', *Am J Cardiol*, vol. 85, no. 11, Jun 1, pp. 1393-1394.

Yan, W, Wu, F, Morser, J & Wu, Q 2000, 'Corin, a transmembrane cardiac serine protease, acts as a pro-atrial natriuretic peptide-converting enzyme', *Proc Natl Acad Sci U S A*, vol. 97, no. 15, Jul 18, pp. 8525-8529.

Yancy, CW, Jessup, M, Bozkurt, B, Butler, J, Casey, DE, Jr., Drazner, MH, Fonarow, GC, Geraci, SA, Horwich, T, Januzzi, JL, Johnson, MR, Kasper, EK, Levy, WC, Masoudi, FA, McBride, PE, McMurray, JJ, Mitchell, JE, Peterson, PN, Riegel, B, Sam, F, Stevenson, LW, Tang, WH, Tsai, EJ, Wilkoff, BL, American College of Cardiology, F & American Heart Association Task Force on Practice, G 2013, '2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines', *J Am Coll Cardiol*, vol. 62, no. 16, Oct 15, pp. e147-239.

Yasue, H, Yoshimura, M, Sumida, H, Kikuta, K, Kugiyama, K, Jougasaki, M, Ogawa, H, Okumura, K, Mukoyama, M & Nakao, K 1994, 'Localization and mechanism of secretion of B-type natriuretic peptide in comparison with those of A-type natriuretic peptide in normal subjects and patients with heart failure', *Circulation*, vol. 90, no. 1, Jul, pp. 195-203.

Yates, JC, Beamish, RE & Dhalla, NS 1981, 'Ventricular dysfunction and necrosis produced by adrenochrome metabolite of epinephrine: relation to pathogenesis of catecholamine cardiomyopathy', *Am Heart J*, vol. 102, no. 2, Aug, pp. 210-221.

Yndestad, A, Damas, JK, Geir Eiken, H, Holm, T, Haug, T, Simonsen, S, Froland, SS, Gullestad, L & Aukrust, P 2002, 'Increased gene expression of tumor necrosis factor superfamily ligands in peripheral blood mononuclear cells during chronic heart failure', *Cardiovasc Res*, vol. 54, no. 1, Apr, pp. 175-182.

Yndestad, A, Damas, JK, Oie, E, Ueland, T, Gullestad, L & Aukrust, P 2006, 'Systemic inflammation in heart failure--the whys and wherefores', *Heart Fail Rev*, vol. 11, no. 1, Mar, pp. 83-92.

Yndestad, A, Holm, AM, Muller, F, Simonsen, S, Froland, SS, Gullestad, L & Aukrust, P 2003, 'Enhanced expression of inflammatory cytokines and activation markers in T-cells from patients with chronic heart failure', *Cardiovasc Res*, vol. 60, no. 1, Oct 15, pp. 141-146.

Yoshida, T, Hibino, T, Kako, N, Murai, S, Oguri, M, Kato, K, Yajima, K, Ohte, N, Yokoi, K & Kimura, G 2007, 'A pathophysiologic study of tako-tsubo cardiomyopathy with F-18 fluorodeoxyglucose positron emission tomography', *Eur Heart J*, vol. 28, no. 21, Nov, pp. 2598-2604.

Zhang, H, Joseph, J, Vasquez-Vivar, J, Karoui, H, Nsanzumuhire, C, Martasek, P, Tordo, P & Kalyanaraman, B 2000, 'Detection of superoxide anion using an isotopically labeled nitrone spin trap: potential biological applications', *Febs Letters*, vol. 473, no. 1, May 4, pp. 58-62.

Zhao, SP & Xu, TD 1999, 'Elevated tumor necrosis factor alpha of blood mononuclear cells in patients with congestive heart failure', *Int J Cardiol*, vol. 71, no. 3, Dec 1, pp. 257-261.

Zheng, L, Nukuna, B, Brennan, ML, Sun, M, Goormastic, M, Settle, M, Schmitt, D, Fu, X, Thomson, L, Fox, PL, Ischiropoulos, H, Smith, JD, Kinter, M & Hazen, SL 2004, 'Apolipoprotein A-I is a selective target for myeloperoxidase-catalyzed oxidation and functional impairment in subjects with cardiovascular disease', *J Clin Invest*, vol. 114, no. 4, Aug, pp. 529-541.

Zois, NE, Bartels, ED, Hunter, I, Kousholt, BS, Olsen, LH & Goetze, JP 2014, 'Natriuretic peptides in cardiometabolic regulation and disease', *Nature Reviews Cardiology*, vol. 11, no. 7, Jul, pp. 403-412.

Zwicker, K, Dikalov, S, Matuschka, S, Mainka, L, Hofmann, M, Khramtsov, V & Zimmer, G 1998, 'Oxygen radical generation and enzymatic properties of mitochondria in hypoxia/reoxygenation', *Arzneimittelforschung*, vol. 48, no. 6, Jun, pp. 629-636.

Addenda and corrigenda

1. Issue of systolic left ventricular function in heart failure patients.

   It is certainly true that the pathophysiology of heart failure would vary in patients with and without substantial impairment of left ventricular systolic function. In fact, in 20% of the heart failure population evaluated in chapter 3, LVEF was >40%.

2. Inter-individual variability in heart failure patients.

   The pathophysiology of heart failure population is multifactorial. It will be ideal to subgroup the patients in regarding of BNP responsiveness. However, the total number of patients recruited in this project is not big enough to do so, and this is also a limitation of the study which has been stated in the thesis.

3. Role of natriuretic peptide fragments in suppression effect.

   So far, systematic study of the effects of different BNP fragments has not been carried out, although it is clear that several fragments are biologically active. Indeed, this could be helpful for understanding of the whole story, answering the question with regard to impaired BNP responsiveness and guiding the development of more effective therapies.

4. Neutrophil stability.

   All experiments have been done immediately and within 3 hours of isolation of neutrophils. Diminution of effect over time has been observed in some cases with fMLP stimulation; therefore, all of the fMLP-related measurements were performed at the very beginning.

5. Is wall stress the only BNP release stimulus?

Although it has been suggested that end diastolic wall stress is the key determinant of BNP release, recent publications implicated other stimuli such as inflammation. Furthermore, the "active BNP_{1-32}" is not the only circulating forms of BNP that has physiological effects (see 3 above). Available commercial BNP assays do not differentiate between different fragments. Subsequent studies using chromatographic assays could be aimed at those aspects.

6. The ASCEND-HF trial may have failed for other reasons.

   The results of the ASCEND-HF trial indicated that "nesiritide (synthesis BNP) cannot be recommended for routine use in the broad population of patients with acute heart failure", because that Nesiritide has no effect either on the rate of death and rehospitalization or dyspnea when used in combination with other therapies. One possibility is lack of effective receptors for nesiritide: - the predominant active natriuretic peptide receptor in heart failure may be NPR-B rather than NPR-A. However, nesiritide was associated with an increase in rates of hypotension suggesting at least some biological activity.

7. Caveat: neutrophils are not equal to other cells.

   This is true. In this particular study we chose neutrophils as the study tissue because: (1) neutrophils have been suggested to play an important role in cardiovascular diseases; (2) they are readily accessible in vivo, unlike myocardium.

8. Basis for recovery could involve change in spectrum of natriuretic peptide fragments.

   This is possible; however, this has not been assessed in this particular study.

9. Non-recovery in TTC suggests that the processes not just BNP-mediated.

   In this research project, we only obtained recovery data from 5 TTC patients, which is

obviously too small a number to draw a final conclusion. Other factors such as severity of the initial attack or treatment could also be involved in the recovery of BNP effects in such patients.

Appendix: Published works related and conducted towards this thesis

Liu, S., Ngo, D.T.M., Stewart, S., Horowitz, J.D. & Chirkov, Y.Y. (2014) B-Type natriuretic peptide suppression of neutrophil superoxide generation: mechanistic studies in normal subjects.
Clinical and Experimental Pharmacology and Physiology, v. 41 (10), pp. 739–743

Liu, S., Ngo, D.T., Chong, C.R., Amarasekera, A.T., Procter, N.E., Licari, G., Dautov, R.F., Stewart, S., Chirkov, Y.Y. & Horowitz, J.D. (2015) Suppression of neutrophil superoxide generation by BNP is attenuated in acute heart failure: a case for 'BNP resistance'.
European Journal of Heart Failure, v. 17 (5), pp. 475-483