



**HATTER**

Institute for Cardiovascular Research in Africa



# The role of melatonin in peripartum cardiomyopathy

**Lauren Nicholson**

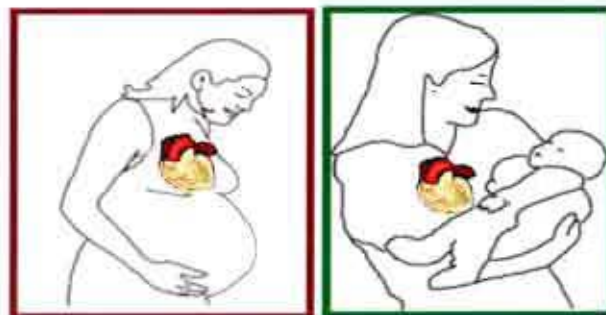
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**Supervisor:**

**Ass Prof. Sandrine Lecour\***

**Co-supervisor:**

**Prof. Karen Sliwa\***

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# **PLAGIARISM DECLARATION**

**Department of Medicine**

**MSc (MED) in Medicine programme 2012-2013**

1. I know that plagiarism is wrong. Plagiarism is to use another's work and pretend its one's own.
2. I have used the American medical association (AMA) convention for citation and referencing. Each contribution to, and quotation in, this thesis from the works of other people has been attributed has been cited and referenced.
3. This thesis is my own work
4. I have not allowed, and will not allow, anyone to copy my work with the intention of passing it off as his or her own work.

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## LIST OF ABBREVIATIONS

$\alpha$	alpha
ACE	angiotensin-converting enzyme
AFMK	N1-acetyl-N2-formyl-5-methoxykynuramine
AMK	N1-acetyl-5-methoxykynuramine
BP	Blood pressure
ATP	adenosine triphosphate
BNP	basic natriuretic peptide
c3OHM	cyclic 3-hydroxymelatonin
CD	cardiac disease during maternity
CDMC	cardiac disease during maternity clinic
CHD	congenital heart disease
CM	cardiomyopathy
CRP	C-reactive protein
DCM	dilated cardiomyopathy
DNA	deoxyribonucleic acid
DSM IV	diagnostic and statistical manual of mental disorders IV
ECG	electrocardiogram
EDPS	Edinburgh postnatal depression scale
EDTA	ethylenediaminetetraacetic acid
EEG	electroencephalogram
ESC	European Society of Cardiology
ETC	Electron transport chain
Fas/Apo-1	apoptosis antigen 1
GSH	Groote Schuur hospital
GSH-Px	glutathione peroxidase
HC	healthy control
HF	heart failure

H <sub>2</sub> O	water
H <sub>2</sub> O <sub>2</sub>	hydrogen peroxide
I/R	ischemia/reperfusion
IL6	Interleukin 6
Jak	Janus Kinase
LV	left ventricle
MDA	malondialdehyde
NO	nitric oxide
NRAS	neuroblastoma rat sarcoma
NT-Pro-BNP	n-terminal basic natriuretic peptide
O	oxygen
O <sub>2</sub> <sup>-</sup>	superoxide anion
OH	hydroxyl radical
ORAC	oxygen radical absorbance capacity
oxLDL	oxidised low density lipoprotein
PPCM	peripartum cardiomyopathy
REM	rapid eye movement
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT	Room temperature
SAFE	Survivor Activating Factor Enhancement
SCN	suprachiasmatic nuclei
Se	selenium
SOD	superoxide dismutase
STAT3	signal transducer and activator of transcription 3
TBARS	thiobarbituric acid reactive substances
USA	United States of America

## ABSTRACT

**Introduction:** Peripartum cardiomyopathy (PPCM) is a heart disease of unknown aetiology emerging in previously healthy women towards the end of pregnancy or first postpartum months. Previous studies have suggested that oxidative stress contributes to the pathogenesis of PPCM. Melatonin is a powerful endogenous antioxidant that can limit the damaging effect of oxidative stress. Melatonin levels are known to be altered in sleep disruption, depression and other cardiac diseases. The aim of this study was to determine if melatonin levels are disrupted in women with PPCM compared to healthy patients. We hypothesised that sleep disruption and depression may contribute to a disruption in their melatonin levels.

**Subjects and Methods:** Pregnant and postpartum healthy control (HC), with (PPCM) or women with other cardiac diseases (CD) were recruited for the study. A sleep quality questionnaire and the Edinburgh postnatal depression scale (EDPS) were performed in all patients to compare their sleeping patterns and depression levels. Daytime and nocturnal salivary melatonin levels were also compared and the serum concentration of cortisol and basic natriuretic peptide (BNP) were measured. To evaluate oxidative stress, a thiobarbituric reactive substances (TBARS) assay was assessed in the plasma of all women.

**Results:** Both BNP levels and oxidative stress were elevated in the postnatal PPCM patients compared to HC and CD women. The PPCM and CD groups demonstrated increased EDPS scores and elevated serum cortisol compared to HC. The postnatal PPCM women also demonstrated delayed, fragmented, inefficient sleep compared to the other women. The PPCM women had significantly higher ( $p < 0.05$ ) postpartum nocturnal melatonin ( $99.8 \pm 8.8 \text{ pg/ml}$ ) levels compared to HC ( $73.4 \pm 8.3 \text{ pg/ml}$ ) and CD ( $77.8 \pm 7.8 \text{ pg/ml}$ ) ( $p < 0.05$ ).

**Interpretation:** The postnatal PPCM women demonstrated an increase in oxidative stress, in salivary nocturnal melatonin, in EDPS scores and sleep disruption, therefore suggesting that disruption in the melatonin synthesis, associated with depression and sleep disturbances, may play an important role in the development of the disease. Further studies will be required to delineate whether melatonin may be considered as a therapeutic target for patients with PPCM.

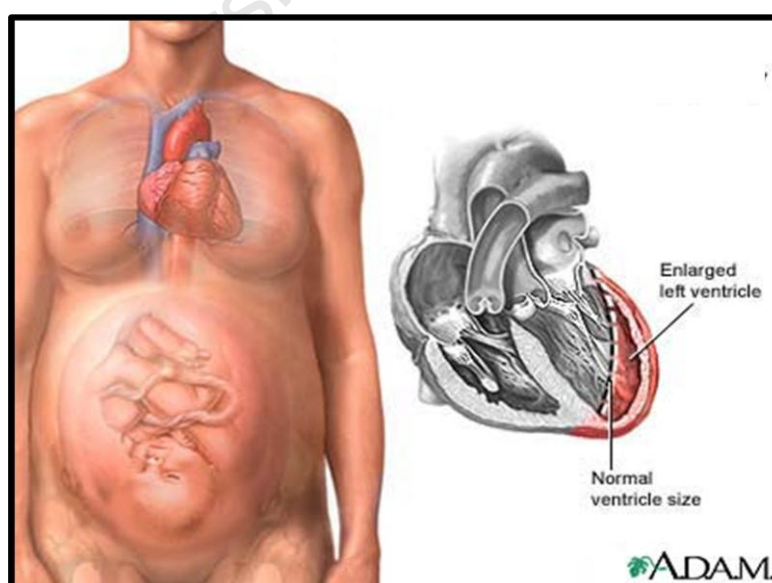
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# CHAPTER 1: INTRODUCTION

## 1. PERIPARTUM CARDIOMYOPATHY (PPCM)

### 1.1 Definition of PPCM

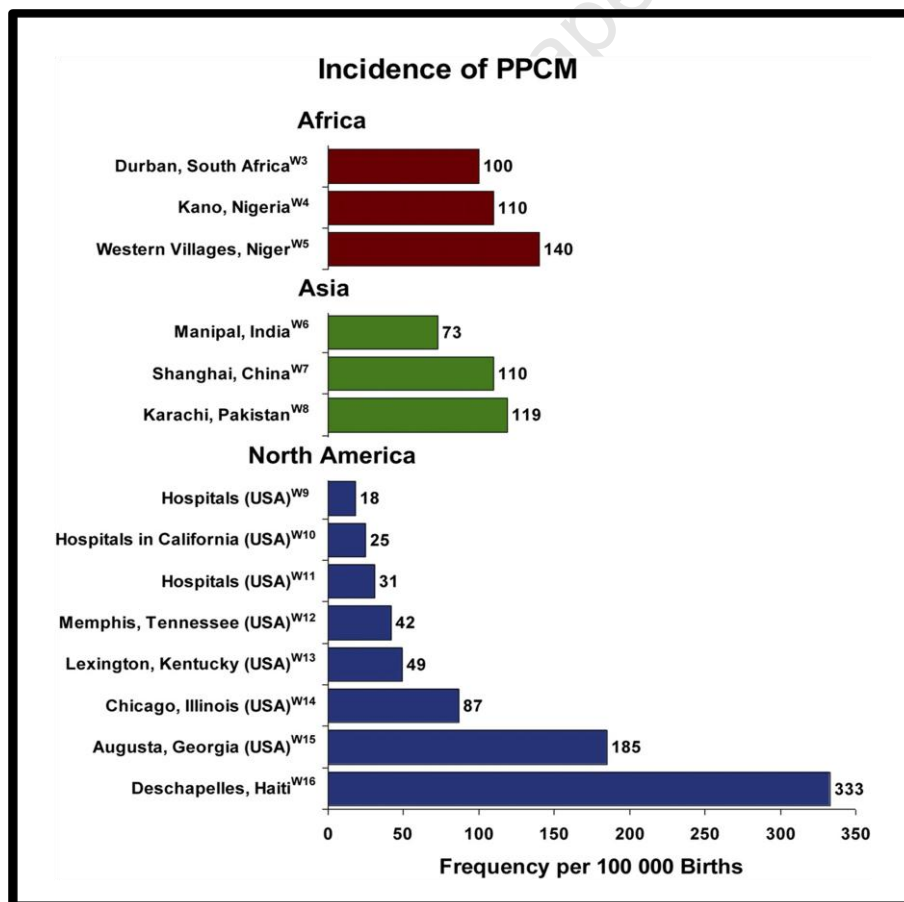
Peripartum cardiomyopathy (PPCM) is a potentially life-threatening heart disease of unknown etiology, emerging in previously healthy women towards the end of pregnancy or during the first postpartum months<sup>1</sup>. Women with PPCM present with initial left ventricular (LV) systolic dysfunction and symptoms of heart failure (**Figure 1**)<sup>2</sup>. The first reference to PPCM in the medical literature was in 1849, but the condition was only recognised as a distinct entity in 1937<sup>3</sup>. The disease affects women with no previous history of cardiac disease and occurs by definition in the final month of pregnancy, or during the first 5 months postpartum<sup>4</sup>. This time interval may, however, lead to under diagnosis, an alternative definition has recently been suggested to extend to "an idiopathic cardiomyopathy presenting with heart failure secondary to left ventricular systolic dysfunction towards the end of pregnancy or in the months following delivery, where no other cause of heart failure (HF) is found"<sup>5</sup>. The diagnosis of PPCM is challenging as, the early detection is hampered by an overlap of symptoms of the normal peripartum period. A final diagnosis can only be made by the exclusion of all other causes of HF<sup>6</sup>. The condition is therefore probably under reported, although current data suggest that it is a leading cause of pregnancy related morbidity and mortality<sup>3</sup>.



**Figure 1.** Diagram showing left ventricular (LV) enlargement, a symptom of peripartum cardiomyopathy. From <http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0001240/>

## 1.2 Epidemiology and prevalence of PPCM

The full epidemiological incidence of PPCM is largely unknown due to the diagnosis being one of exclusion<sup>6</sup>. There is a large geographical and racial discrepancy in the occurrence of PPCM in different populations<sup>3</sup>. The reported incidence of PPCM per 100000 women in Africa, Asia and the United States of America (USA) is shown in **Figure 2**<sup>6</sup>. The current literature reports the incidence to be far higher in developing countries, for example 1 in 300 in Haiti and 1 in 1000 in South Africa and China, compared to developed countries such as North American and Europe with 1 in 2500-4000<sup>5</sup>. African decent is suggested as a possible risk factor. The disease affects regions with large African populations such as Nigeria, South Africa and Haiti<sup>3</sup>. Regions in developed countries with large numbers of socio-economically disadvantaged population groups, such as Georgia, compared to the more wealthy state of California in USA, also show higher frequencies of PPCM<sup>6</sup>.

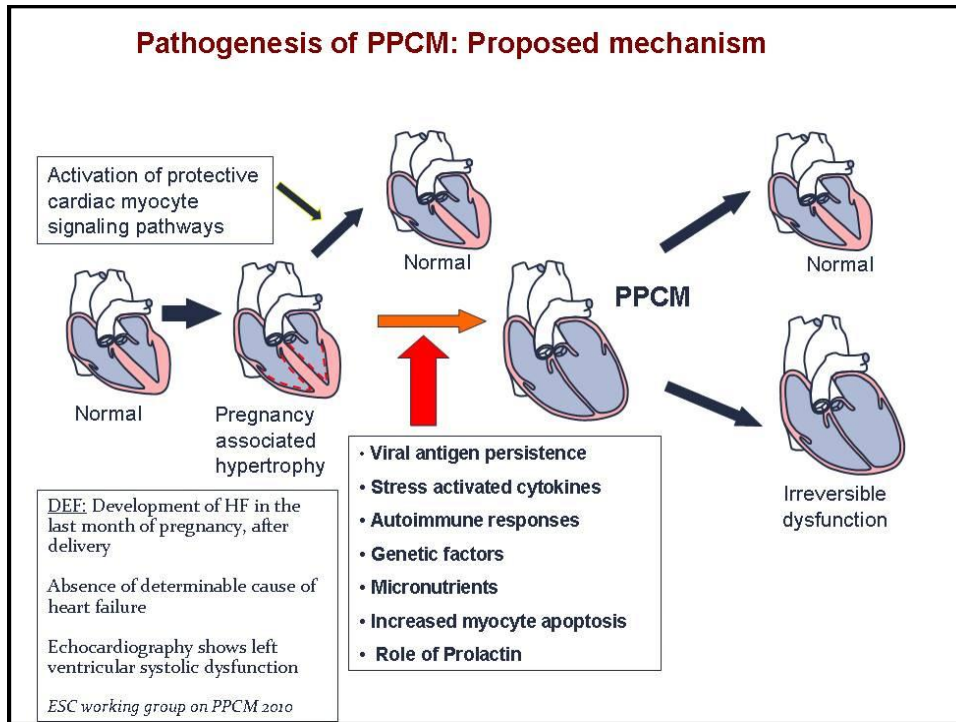


**Figure 2.** The reported incidence of peripartum cardiomyopathy in North America, Asia and Africa. The condition remains relatively rare in developed regions such as California and is more common in developing regions such as Haiti, China and South Africa. From Blauwet et al<sup>6</sup>.

### 1.3 Pathophysiology, symptoms and diagnosis of PPCM

Major compensatory changes are made by the maternal heart to accommodate the demands of pregnancy and lactation<sup>7</sup>. During pregnancy, women experience a reversible cardiac hypertrophy and reduced relaxation of diastolic function but, in healthy women, this regresses to normal following childbirth (**Figure 3**)<sup>8</sup>. In the case of PPCM, the maternal heart does not regress to normal and deteriorates<sup>9</sup>. It has been suggested that anti-angiogenic signalling, during pregnancy and post partum may contribute to the development of PPCM. Anti-angiogenic factors in the placenta secretes vascular endothelial growth factor (VEGF) inhibitors such as soluble Vascular endothelial growth factor receptor 1 (Flt1)<sup>10</sup>, high levels of circulating levels of Flt1 are known to lead to cardiac dysfunction, particularly in mice lacking pro-angiogenic Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ). Women who develop PPCM have been shown to have high levels of sFlt1<sup>10</sup>.

The diagnosis of PPCM is often delayed due to the variability of the clinical manifestations, as well as similarities to other types of HF secondary to cardiomyopathy (CM)<sup>11</sup>. Patients with PPCM often present with elevated LV end-diastolic pressure due to systolic dysfunction and a diagnosis can only be made once all other causes of HF have been excluded<sup>5</sup>. The early signs and symptoms may be mild or mirror those of normal pregnancy<sup>5</sup>. The presenting symptoms, which include fatigue and dyspnoea (shortness of breath) on mild exertion, are often mistaken for other conditions such as pneumonia or complications related to delivery, causing a delay in referral to cardiologists<sup>11</sup>. Other symptoms include pedal oedema, orthopnoea, dizziness and palpitations<sup>5</sup>. A list of common presenting signs and symptoms is shown in **Table 1**<sup>11</sup>. An electrocardiogram (ECG) and echocardiography are useful in excluding other forms of heart failure which may help the diagnosis<sup>5</sup>. The ECG often includes changes such as abnormal rhythms, including skipped beats and arrhythmias (**Table 1**)<sup>11</sup>. The echocardiogram demonstrates changes including LV dilation, as well as reduced ejection fraction (EF)<sup>11</sup>. The B-type natriuretic peptide (BNP) and N-Terminal BNP (NT-pro BNP) are often elevated in women with PPCM<sup>5</sup>. BNP has an important function in the regulation of cardiovascular and renal homeostasis, as well as in the regulation of fatty acid metabolism and body weight<sup>12</sup>. Studies have demonstrated a positive correlation between the extent of cardiac damage and BNP concentration<sup>12</sup>. BNP is therefore a useful tool in the diagnosis and treatment monitoring of PPCM<sup>12</sup>. Serum microparticles have recently been shown to be a potentially useful tool in the diagnosis of PPCM.<sup>13</sup>



**Figure 3.** Proposed mechanism for the pathogenesis of PPCM. During pregnancy the heart undergoes hypertrophic changes. In normal women the heart returns to normal following delivery, however in some women possibly due to factors such as an increase in oxidative stress or other factors leads to heart failure. Following the development of PPCM some women return to normal, however some suffer irreversible cardiac dysfunction adapted from Sliwa et al <sup>29</sup>.

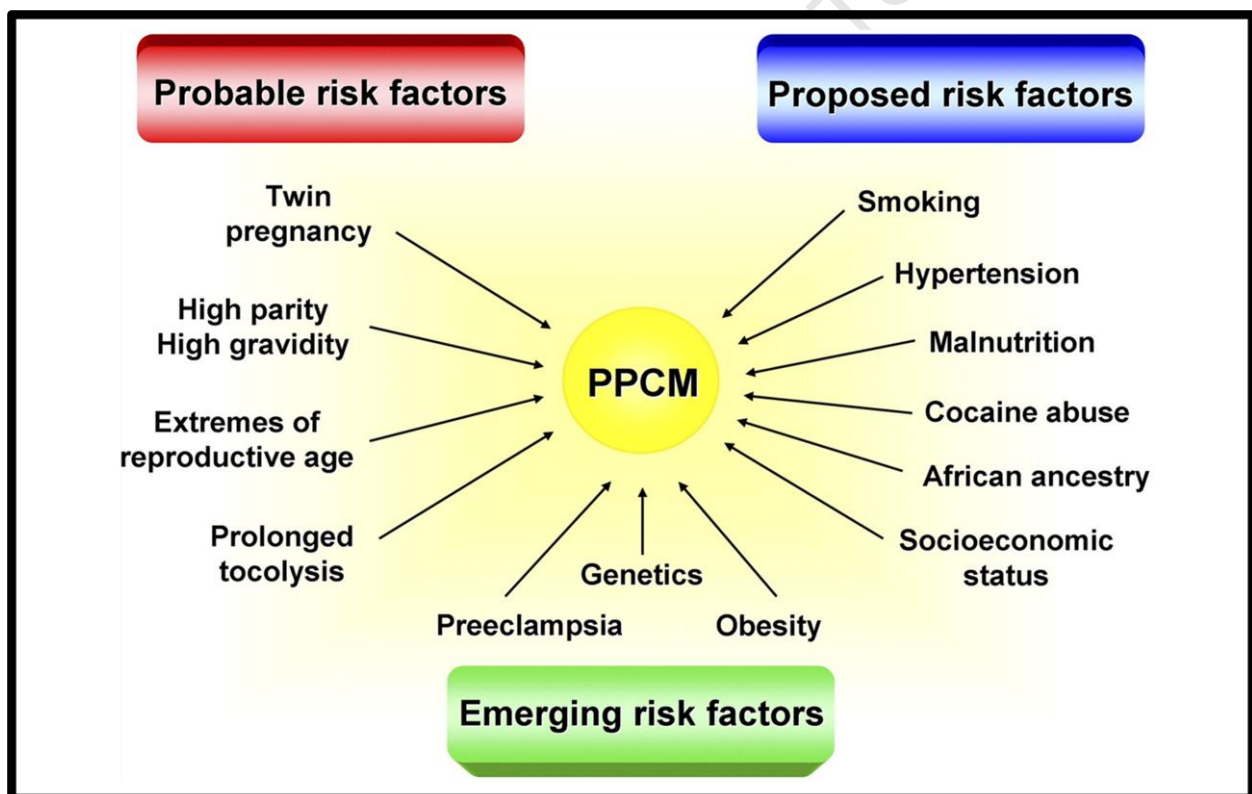
**Table 1.** Signs and symptoms of peripartum cardiomyopathy. From Hilfiker-Kleiner et al.<sup>11</sup>.

<b>Symptoms and signs which, in the postpartum period, suggest PPCM</b>
● Lassitude and exhaustion
● Dyspnea
● Paroxysmal nocturnal dyspnea
● Wet crepitations over the lung fields
● Shadowing on the chest X-ray
● Leg edema
● Nocturia
● Palpitations or missed beats
● Newly arising repolarization abnormalities on the ECG
● Arrhythmias on the ECG
● Systolic murmur
● Impaired left ventricular function
● New-onset secondary mitral regurgitation
● Arterial emboli
● Cerebral emboli

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## 1.4 Risk factors and proposed mechanisms of PPCM

The current data is suggestive that a complex interaction between genetic factors, combined with the physiological adaptation of pregnancy and childbirth, contributes to the development of PPCM<sup>14</sup>. The exact causative factors of PPCM are unclear. A number of factors have been suggested to contribute to the condition, including pregnancy-related factors such as age, number of children, toxolytic ingestion and malnutrition<sup>5</sup>. The other suggested factors include the general cardiovascular risk factors such as smoking, hypertension and diabetes<sup>5</sup>. A diagram of probable, proposed and emerging risk factors for the development of PPCM is shown in **Figure 4**<sup>6</sup>. PPCM likely results from a complex interaction between a combination of multiple factors, including socio-economic and pregnancy associated factors<sup>8</sup>.



**Figure 4.** The proposed, probable and emerging risk factors which possibly contribute to the development of peripartum cardiomyopathy From Blauwet et al<sup>6</sup>.

### **1.4.1 Role of genetics in PPCM**

The geographical and racial discrepancies, as well as anecdotal evidence noting familial clustering in PPCM, is suggestive of a genetic cause. However, this has not been established<sup>3</sup>. Some reports suggest that the cases in which related women have been diagnosed with PPCM are in fact cases of dilated cardiomyopathy (DCM) unmasked by the hemodynamic stress of pregnancy<sup>15</sup>. The presentation of PPCM is similar to DCM<sup>11</sup>. These studies also suggest that PPCM may be part of the DCM spectrum, with related mutations<sup>6</sup>. A model of DCM in mice who have an over-expression of the alpha ( $\alpha$ ) subunit of the protein Gq in cardiac tissue have been shown to develop pregnancy-induced cardiomyopathy<sup>8</sup>. This has, however, not been shown to translate into human women who develop PPCM<sup>8</sup>. A genetic component cannot be fully excluded, although most of the evidence points to the risk factors being a combination of other factors rather than hereditary<sup>5</sup>.

### **1.4.2 Role of inflammation in PPCM**

Inflammation is a biological response of the immune system on vascular tissues to stimuli such as damaged cells, irritants and pathogens<sup>16</sup>. There is evidence to suggest that inflammation has a role in the pathophysiology of PPCM<sup>7</sup>. The activation of pro inflammatory cytokines and elevation of C-reactive protein (CRP) is a major characteristic of HF<sup>3</sup>. CRP is a group of acute phase proteins which specifically bind to phosphocholine and recognize target membranes of reactive and apoptotic cells<sup>4</sup>. Forster et al. investigated whether such reactive proteins CRP and tumour necrosis factor alpha (TNF $\alpha$ ), as well as apoptosis antigen 1 (Fas/Apo-1), could predict the outcome in patients with PPCM<sup>17</sup>. The study found that the plasma markers for inflammation, as well as Fas/Apo-1, were increased significantly and in direct proportion to an increase in LV size and a decrease in EF<sup>17</sup>. A study by Sliwa et al., in South Africa, documented the clinical outcomes relative to inflammatory markers in 100 PPCM patients<sup>4</sup>. The CRP and the inflammatory cytokine interleukin-6 (IL6) levels were significantly raised in more than half of the women who developed PPCM<sup>4</sup>. The study also suggested that low total cholesterol was a predictor of poor outcome, larger LV dimensions and lower EF<sup>4</sup>.

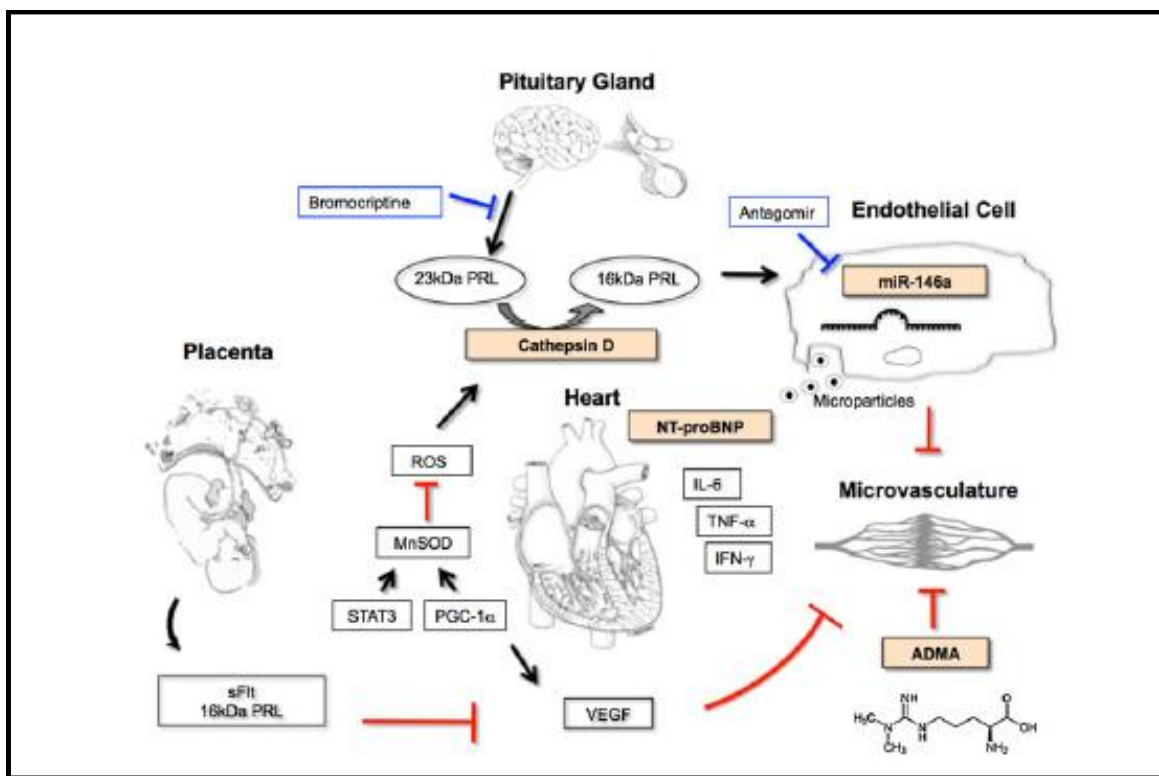
Sliwa et al. performed a study to further understand the mechanisms underlying PPCM in humans<sup>17</sup>. The baseline serum markers relating to apoptosis, oxidative stress remodelling, inflammation and cardiac function were analysed in women with PPCM and healthy controls<sup>17</sup>. The patients were followed up at 6 months and were either categorised as improved or not improved<sup>17</sup>. The majority of the markers of oxidative stress, inflammation apoptosis and cardiac stress were higher in the PPCM

group compared to healthy controls. The results also suggested a correlation between oxidative stress, inflammation and prolactin<sup>17</sup>. Baseline NT-pro BNP levels were significantly higher in the PPCM group that did not improve after 6 months<sup>17</sup>.

### 1.4.3 Role of prolactin in PPCM

Hilfiker-Kleiner et al. highlighted the fact that the transcription factor signal transducer and activator of transcription 3 (STAT3) functions in a diverse number of physiological situations, including heart function with what appears to be opposite functions, including proliferation and apoptosis<sup>18</sup>. The down regulation of STAT3 is associated with late-stage heart failure and the activation of STAT3 is associated with the survival of cardiomyocytes and angiogenesis<sup>18</sup>. The study by Hilfiker-Kleiner et al. included mice with a cardiomyocyte specific deletion of STAT3, which are sensitive to cardiac insult<sup>18</sup>. Oxidative stress is enhanced in these mice which leads to cardiac cathepsin-D mediated cleavage of prolactin into a 16kDa pro apoptotic form. As a consequence, these mice suffer high mortality, heart failure and a decrease in cardiac angiogenesis which are hallmarks of PPCM<sup>18</sup>. Hilfiker-Kleiner et al. also demonstrated a reduction in the STAT3 protein in the heart tissue of women with PPCM in comparison to healthy controls<sup>19</sup>. The study also demonstrated an increase in oxidized low-density lipoprotein (oxLDL) in the patients with PPCM<sup>19</sup>. The down regulation of STAT3 and the fragmentation of the 23kDa breastfeeding hormone, prolactin, into the 16kDa pro apoptotic form is shown in **Figure 5**. A separate study also determined that a failure to reduce oxLDL and prolactin in women diagnosed with PPCM was an predictor of poor outcome<sup>20</sup>.

In light of the prolactin research, Twickler et al. proposed that the matrix metalloproteinase of trophoblastic cells could facilitate the cleavage of prolactin and possibly contribute to PPCM and pre-eclampsia<sup>21</sup>. Pre-eclampsia, like PPCM, is a pregnancy specific condition characterised by hypertension and proteinuria<sup>21</sup>. There is a high co-morbidity between PPCM and pre-eclampsia and further research is needed to determine whether there is indeed a connection<sup>21</sup>. In Africa, low plasma levels of the antioxidant selenium (Se) level have been reported as a possible risk factor for PPCM<sup>22</sup>. Combs et al. suggested that Se deficiency plays a role in PPCM, but not as a direct causative factor<sup>22</sup>. These studies suggest that oxidative stress is a significant contributor to the development of PPCM (See Chapter 2:oxidative stress).

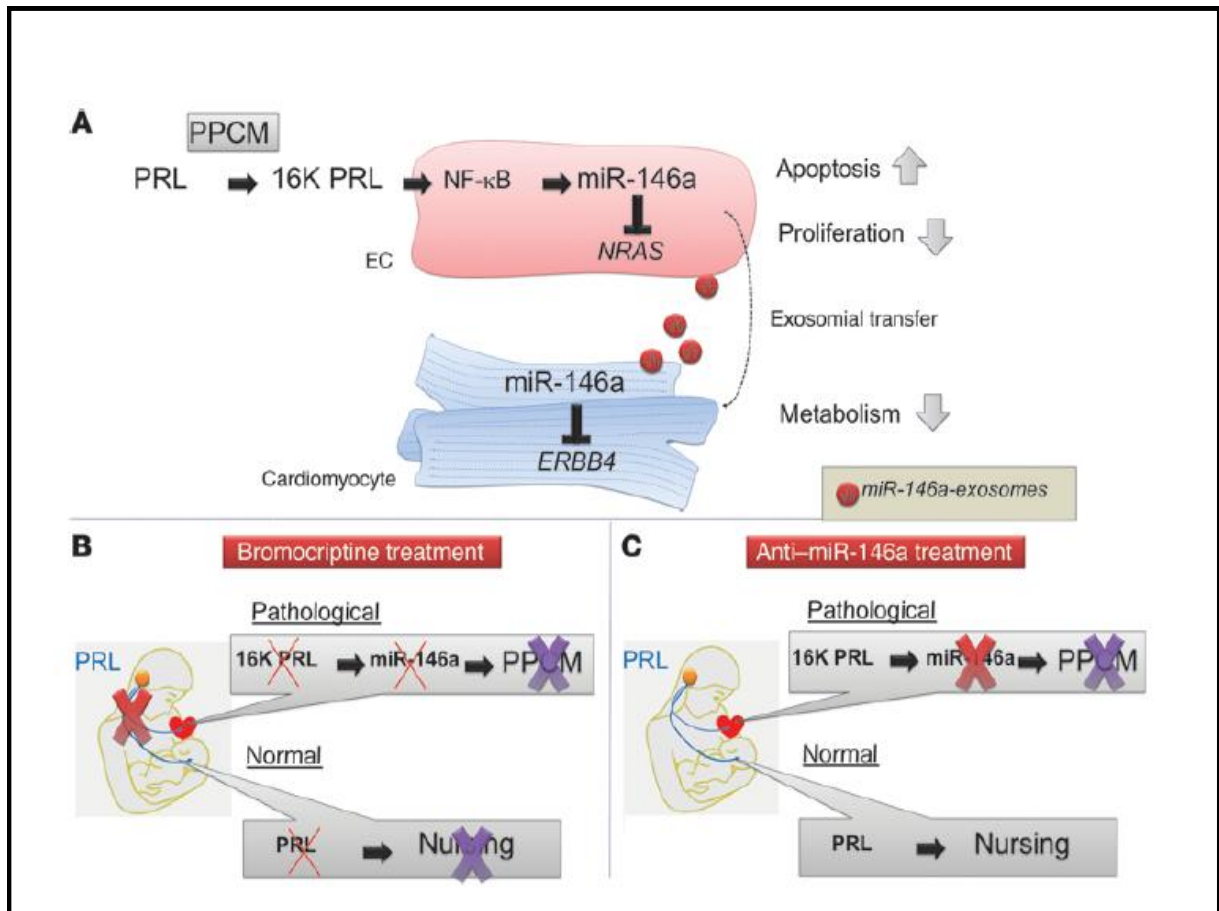


**Figure 5.** Role of prolactin in PPCM and the mechanism of action of bromocriptine. Bromocriptine blocks the production of 23kDa prolactin from the pituitary gland. This prevents the cleavage of prolactin into the 16kDa pro apoptotic form and this likely leads to an improvement in women with PPCM. Recently the inhibition of microRNA145a a downstream consequence of the 16kDa prolactin is a promising therapy. From Haghikia et al. <sup>23</sup>.

## 1.5 Treatment and prognosis

A certain percentage of women with PPCM do recover and their LV function normalises (**Figure 4**)<sup>24</sup>. The remaining portion often suffers from major adverse effects following PPCM, including mortality in up to 25% of women with PPCM<sup>24</sup>. In women who recover, there a risk for reoccurrence remains in future pregnancies and women with previous PPCM diagnosis should be monitored in subsequent pregnancies<sup>25</sup>. Poor outcome was particularly associated with women whose LV function had not returned to normal<sup>2</sup>. Early diagnosis of the condition is essential for the initiation of appropriate medical care and counselling for future prognosis<sup>19</sup>.

Currently, PPCM is treated with standard heart failure drugs, including angiotensin-converting enzyme (ACE) inhibitors, diuretics, aldosterone antagonists, and beta-blockers<sup>19</sup>. Special attention should be given to safety if ACE inhibitors are administered during pregnancy, due to their potential teratogenic effects<sup>3</sup>. Bromocriptine, an inhibitor of prolactin release from the pituitary gland, is a promising treatment for PPCM<sup>19</sup>. The mechanism of action of bromocriptine in the treatment of PPCM is shown in **Figure 5**<sup>19</sup>. A prospective, proof-of-concept pilot study was performed at a single centre in South Africa by Sliwa et al., comparing the use of bromocriptine and standard HF drugs in the treatment of PPCM, over a period of 8 weeks<sup>26</sup>. The results of the study found no significant difference in baseline characteristics in serum 16-kDa prolactin levels and cathepsin D activity<sup>26</sup>. The group who received bromocriptine did, however, display a greater LV EF and a lower mortality rate than the group on standard heart failure treatment<sup>26</sup>. When bromocriptine is used, the entire prolactin cascade is inhibited which excludes breastfeeding (**Figure 6**)<sup>27</sup>. A new promising target for the treatment of PPCM, further downstream the prolactin cascade is the microRNA-146a (**Figure 6**)<sup>27</sup>. MicroRNAs are short, non-coding small sequences which post-transcriptionally alter gene expression. Recently the 16Kda has been shown to induce microRNA-146a expression in endothelial cells, which attenuated angiogenesis through down regulation of neuroblastoma rat sarcoma (*NRAS*) (**Figure 6**)<sup>27</sup>. The inhibition allows the maintenance of normal nursing due to the downstream harmful products of the 16kDa fragment of prolactin being inhibited rather than the beneficial 23kDa form<sup>27</sup>.



**Figure 6.** Role of miRNA-146a in PPCM and proposed alternative treatment by maintaining normal prolactin functions. **Figure 6A:** Cleavage of prolactin which increases miR-146a expression. **Figure 6B:** Blocking prolactin completely by use of bromocriptine eliminates the 16 kDa pro apoptotic prolactin, but stops breastfeeding. **Figure 6C:** Use of anti-miR-146a in less severely affected patients may improve PPCM recovery, while maintaining breastfeeding. From Halkien et al.<sup>27</sup>.

## 2. OXIDATIVE STRESS

### 2.1 Definition of oxidative stress

Oxidative stress involves a shift towards the pro-oxidant side of the pro-oxidant/antioxidant balance<sup>28</sup>. This functions in a wide range of pathological progress and is increasingly being recognized as a major contributing factor in a large number of diseases<sup>28</sup>. Oxidative stress is caused by free radicals, unstable atoms or molecules which have an unpaired electron and are reactive due to their tendency to bind in order to complete the pair<sup>29</sup>. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are two classes of free radicals in the oxidative stress pathway<sup>30</sup>. The ROS family includes, the superoxide anion ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) and the hydroxyl radical ( $OH$ )<sup>28</sup>. ROS are beneficial in a variety of cellular physiological processes, including their bactericidal properties<sup>30</sup>. When there is an excess of ROS, the beneficial effects are negated by the negative effects of oxidative stress and include protein denaturation, lipid fragmentation as well as the destruction of enzymes and deoxyribonucleic acid (DNA)<sup>30</sup>. RNS is a by-product of normal endothelial metabolism<sup>28</sup>. The nitrogen oxide (NO) radical also has many helpful and harmful processes<sup>28</sup>. The molecule can exist in various forms as fully reduced nitrogen or fully oxidised nitrate<sup>28</sup>. Peroxynitrite is formed when  $O_2^-$  and NO are produced in near equimolar ratio<sup>31</sup>. Peroxynitrite has multiple damaging effects, including DNA damage, as well as lipid peroxidation<sup>31</sup>. Many factors can influence the overproduction of superoxide anion radical in both the cytosol and mitochondria<sup>32</sup>. Following the overproduction of superoxide anions and the oxidation of essential molecule nitro oxidative stress, peroxynitrite forms and causes cellular damage<sup>32</sup>. First, peroxynitrite has direct toxic effects leading to lipid peroxidation, protein oxidation, and DNA damage<sup>32</sup>. Secondly, this leads to the induction of transcription factors which induce an inflammatory cascade and chronic inflammation<sup>32</sup>.

The shift in the oxidative balance occurs when the production of free radicals exceeds the antioxidant capacity (pathways which provide protection against the harmful effects of free radicals)<sup>33</sup>. The reaction which ROS and RNS produce, is a reduction-oxidation (redox) reaction<sup>34 35</sup>. During a redox reaction a pair of electrons from the oxidized molecule are transferred to the reduced molecule (ROS or RNS) and energy is released<sup>35</sup>.

RNS and ROS have short half lives and are therefore difficult to measure directly<sup>33</sup>. Oxidative stress can be measured indirectly, either by a decrease in total antioxidant capacity or by the estimation of the products of oxidation of lipids or DNA such as malondialdehyde (MDA)<sup>33</sup>.

## 2.2 Oxidative stress during pregnancy and postpartum

The oxidative process during pregnancy has a regulatory function<sup>36</sup>. The regulation of vascular changes, as well as the uterine and cervical tone in the maternal body, is dependent on the mechanisms involving the metabolic pathways of ROS and RNS<sup>37</sup>. At the start of pregnancy, an inflammatory cascade is activated which allows the formation and invasion of the foreign trophoblastic material into the maternal tissues<sup>28</sup>. These pathways also control normal and pathologic embryogenesis<sup>28</sup>. The production of NO and RNS are essential at this point, by modulating the metalloproteinase which functions to remodel the uterine extracellular matrix<sup>37</sup>. The hormone oestrogen mediates the regulation of the balance of the pro-oxidative and anti-oxidative molecules guarding this process<sup>28</sup>. An increase in oxidative stress during pregnancy can be characterised by enhanced lipid peroxidation and the circulation of lipid hydroperoxides and MDA<sup>28</sup>.

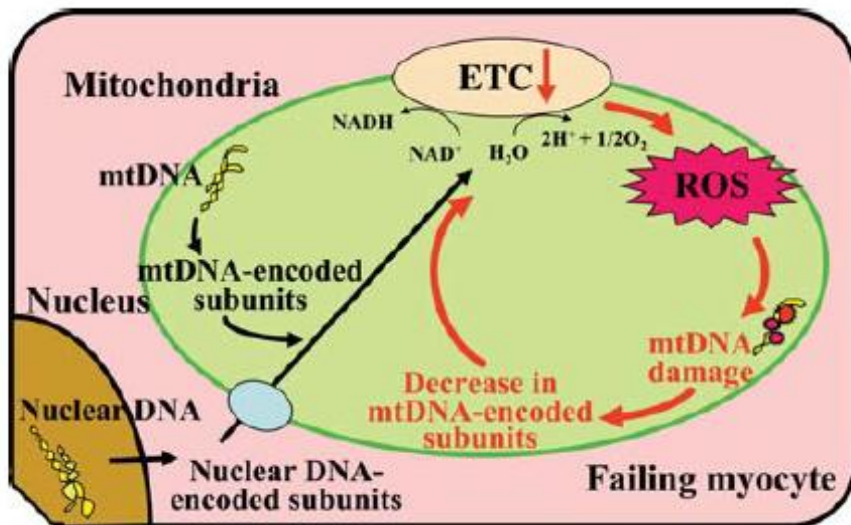
The placenta is rich in iron, a transitional metal which is important in the production of free radicals<sup>38</sup>. The increase in oxidative stress in healthy women peaks around the second trimester<sup>38</sup>. When the pregnancy becomes advanced, disruption in this oxidative balance can lead to an inappropriate activation of the inflammatory cascade, which produces harmful effects, including premature labour and complications such as pre eclampsia<sup>28</sup>. A study by Mittal et al. compared the levels of 3 oxidative stress markers, glutathione peroxidase, superoxide dismutase and MDA, as well as the 2 antioxidants (vitamin C and lycopene) in healthy and pre-eclamptic pregnant women<sup>39</sup>. The study observed that women with pre-eclampsia had higher levels of oxidative stress markers and significantly lower antioxidants compared to healthy women<sup>39</sup>.

### 2.3 Oxidative stress and cardiovascular disease

Oxidative stress plays a central role in the pathogenesis of cancer, atherosclerosis, aging and other chronic diseases<sup>40</sup>. ROS and oxidative damage are known contributors to the damage associated with ischemia-reperfusion (I/R) injury<sup>40</sup>. The lack of oxygen to the tissues, as observed in ischemia, is damaging. However the restoration of blood is equally destructive as it is associated with an excessive production of free radicals<sup>41</sup>. Atherosclerosis is a chronic vascular disease in which inflammation and oxidative stress are implicated as major causative factors of the disease<sup>41</sup>. This leads to excessive deposits of oxLDL in large arteries due to inflammation and oxidative stress<sup>41</sup>.

Oxidative stress is known to be enhanced in myocardial remodelling and failure<sup>42</sup>. Established risk factors of cardiac disease such as hypertension, high fat diet and smoking are all associated with an increase in oxidative stress<sup>40</sup>. Sustained increases in oxygen radical production in the mitochondria may lead to a destructive cycle of mitochondrial DNA damage, as well as functional decline, further ROS generation and cellular injury (**Figure 7**)<sup>42</sup>. The additional increase in ROS induces myocyte hypertrophy, apoptosis, and interstitial fibrosis by activating matrix metalloproteinases<sup>42</sup>.

As mentioned previously, oxidative stress is a major emerging contributor to the development of PPCM<sup>19</sup>. In women with PPCM, an increase in oxidative stress, as well as a reduction in cardioprotective STAT3, leads to prolactin cleavage and contributes to the LV dilation associated with PPCM<sup>19</sup>. The levels of oxLDL in women with PPCM are also known to be increased<sup>19</sup>.



**Figure 7.** Schematic representation of a link between reactive oxygen species (ROS), mitochondrial DNA damage and respiratory chain dysfunction in the mitochondria. Mitochondrial ROS generation may lead to a catastrophic cycle of mitochondrial functional decline, further ROS generation, and cellular injury. ETC, electron transport chain in a failing myocyte. From Tsutsui et al.<sup>42</sup>.

## 2.4 Endogenous and exogenous antioxidants

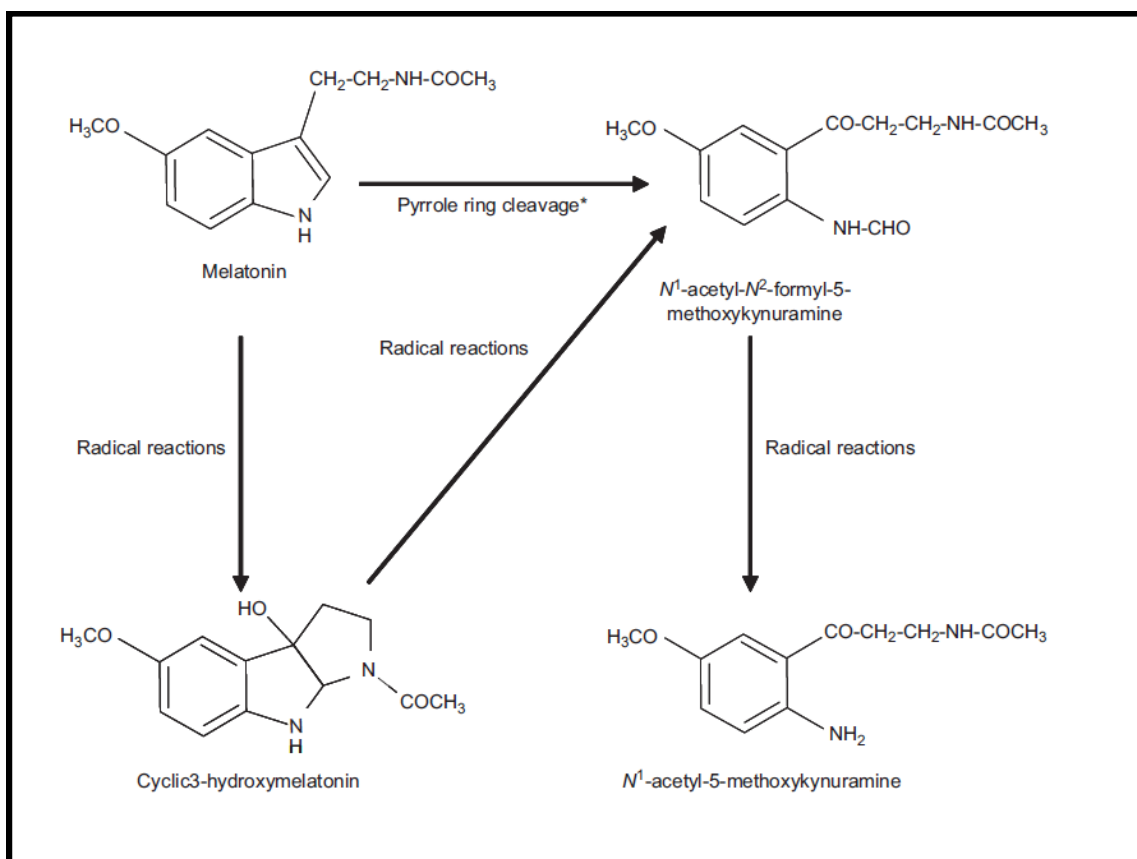
An endogenous antioxidant defence system protects cells from oxidative damage and may act via enzymatic or non-enzymatic pathways<sup>28</sup>. The non-enzymatic antioxidants, including Vitamin A, E and C, as well as selenium, help to protect tissue against damage by preventing the initial formation and blocking the propagation of ROS<sup>28</sup>. The most effective oxidant scavengers include superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-Px). Antioxidants have been shown in experimental studies to protect against the harmful effects of oxidative stress, however this has not been translated to randomised clinical trials<sup>40</sup>. Antioxidants may still prove a potential therapeutic tool in the prevention and treatment of cardiovascular disease<sup>40</sup>.

Exogenous antioxidants may be consumed in the form of supplements or dietary intake. Individuals who consume a typical western diet, high in trans fats and grains and low in fruits and vegetables are at risk for trace element and vitamin deficiency<sup>29</sup>. A diet rich in fruits and vegetables has been shown to reduce the risk of cardiovascular disease<sup>35</sup>. The mechanism by which this protection is conferred has been difficult to prove and the antioxidant effect has been suggested<sup>35</sup>. Studies in Europe have demonstrated a low plasma concentration of micronutrients (iron, selenium, zinc, vitamins B1, B6, C, A and E) in healthy individuals<sup>29</sup>. Vitamin E has been extensively studied in large prevention trials, but no benefit has been demonstrated<sup>40</sup>. A randomised placebo-controlled supplemented women at risk for pre-eclampsia with high doses of vitamins E and C (VIP trial)<sup>43</sup>. The supplementation did not decrease the risk of pre-eclampsia in these women and the risks of high doses of these antioxidants during pregnancy were concluded to be unjustified<sup>43</sup>. The conclusion that the antioxidants do not contribute to the protection may be premature, as the physiological dose may be incorrect or the wrong type of antioxidants are being used<sup>35</sup>. An antioxidant which has shown promise in the reduction of ROS is melatonin<sup>44</sup>.

### 3. MELATONIN

#### 3.1 Definition and structure of melatonin

Melatonin is a methoxyindole hormone synthesized and secreted by the pineal gland from tryptophan, taken up from the circulation and converted to melatonin by a 2 step enzyme transfer<sup>45</sup>. Melatonin is well known as a "sleep hormone" and acts as a time giver, essential to both the timing and onset of circadian rhythm and reproduction<sup>46</sup>. Melatonin is also a powerful natural antioxidant, able to easily reach cellular and sub cellular compartments, due to its small size and amphiphilic nature<sup>47</sup>. Melatonin acts as an endogenous free radical scavenger and broad spectrum antioxidant<sup>47</sup>. In the presence of oxidants, melatonin generates the powerful antioxidant products, cyclic 3-hydroxymelatonin (c3OHM) and N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK)<sup>48</sup>. The free radical scavengers c3OHM and AFMK both lead to the formation of N1-acetyl-5-methoxykynuramine (AMK), which in turn is also a free radical scavenger (**Figure 8**)<sup>48</sup>. Nutritional, environmental, and chemical factors can induce the overproduction of the superoxide anion radical in both the cytosol and mitochondria<sup>49</sup>. Melatonin is secreted by the hypothalamic circadian pacemaker which is synthesised from serotonin in the pineal gland under limiting influence of norepinephrine<sup>50</sup>. Mitochondria, the powerhouse of the cell, have a high intracellular level of melatonin<sup>47</sup>. Mitochondria are particularly important in the formation of ROS which, when produced in excess, damage the mitochondrial lipids proteins and DNA<sup>47</sup>. Cardiolipin, a phospholipid on the inner membrane of the mitochondria, is known to be involved in several mitochondrial bio-energetic processes such as apoptosis<sup>47</sup>. Changes to cardiolipin structure has been associated with mitochondrial dysfunction and oxidative stress<sup>47</sup>. Melatonin has been reported to protect the mitochondria by preventing the cardiolipin associated oxidative damage<sup>47</sup>.

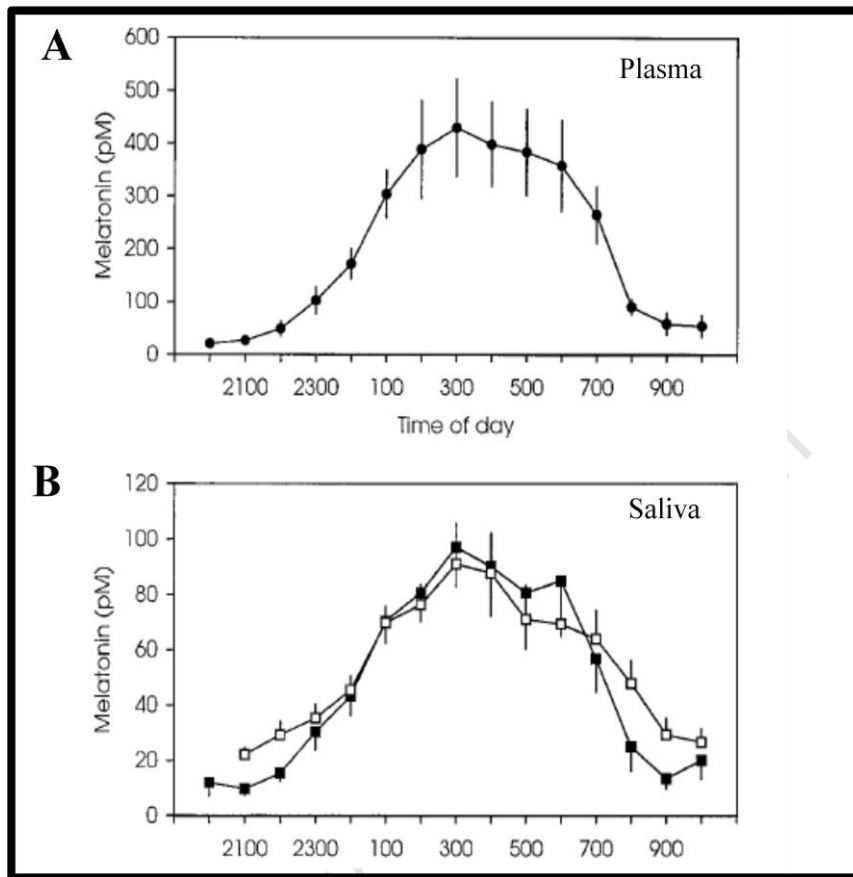


**Figure 8.** The antioxidant cascade of melatonin. When melatonin interacts with oxidants cyclic 3-hydroxymelatonin (c3OHM) and *N*<sup>1</sup>-acetyl-*N*<sup>2</sup>-formyl-5-methoxykynuramine (AFMK) is generated. Both c3OHM and AFMK are powerful antioxidants themselves, leading to the formation of *N*<sup>1</sup>-acetyl-5-methoxykynuramine (AMK). From Russel et al.<sup>48</sup>.

### 3.2 Melatonin rhythm in humans

Human average melatonin levels are lowest during daytime hours and peak around 2-4am (**Figure 9**)<sup>50</sup>. **Figure 9A** shows the plasma melatonin levels in humans and **Figure 9B** shows the salivary melatonin levels which correlate to the plasma, although at a lower concentration<sup>50</sup>. Melatonin secretion is lowered by pulses of bright light at night, and by a variety of drugs such as beta blockers<sup>49</sup>. Melatonin secretion has also been shown to be lowered in humans who consume caffeine on a regular basis<sup>49</sup>. Melatonin is generally not released during the day and increases 2 hours before sleep onset and declines during the morning hours. Melatonin should be measured under controlled light conditions as even dim light can suppress melatonin release<sup>50</sup>.

There are individual differences in the timing and onset of the release of melatonin<sup>51</sup>. Humans can be classified as either "morning people", these individuals tend to be more fatigued at night and rise earlier<sup>51</sup>, whereas "evening people" tend to be more active at night and rise later in the morning, with more fatigue<sup>51</sup>. Data suggest this is due to individual differences in the timing and onset of melatonin release<sup>51</sup>. The use of exogenous melatonin supplementation has previously been reported to induce drowsiness and sleep and has been suggested to be beneficial in alleviating sleep disturbances and nocturnal awakenings<sup>52</sup>. The influence of eye colour on the suppression of melatonin secretion of bright light was studied by Higuchi et al.<sup>53</sup>. The study compared individuals with light eye colour (green or blue) compared to dark eye colour (brown)<sup>53</sup>. The study found that salivary melatonin was significantly more suppressed in individuals with light eye colour compared to dark<sup>53</sup>. These data suggest a difference in melatonin secretion between individuals with different ethnicity/eyecolour<sup>53</sup>.

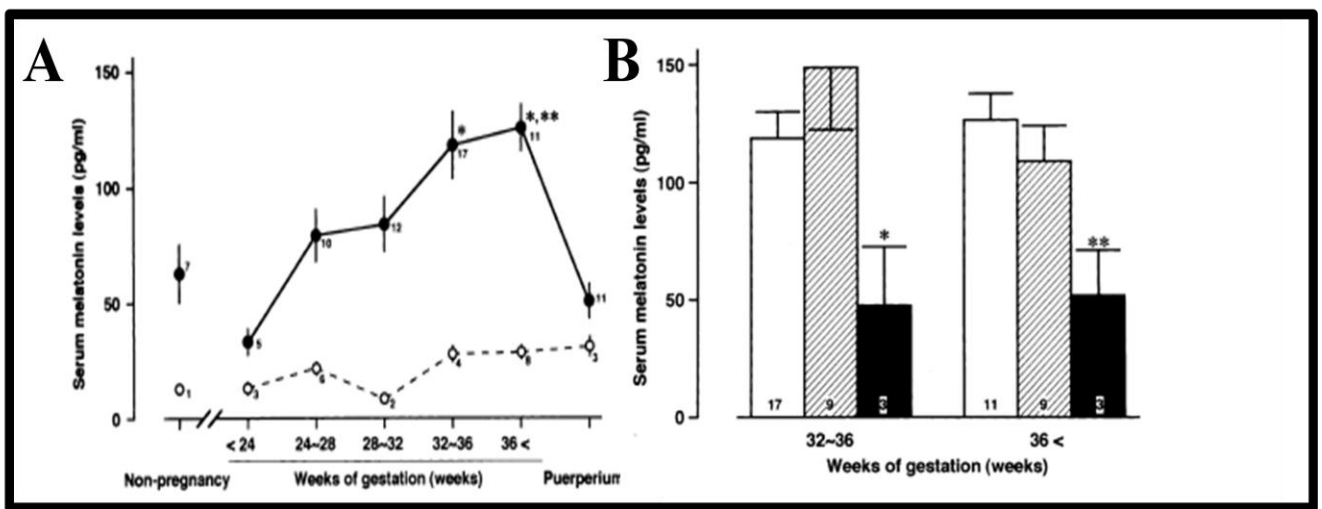


**Figure 9.** Figure 9A: the plasma melatonin levels in 10 healthy humans between the hours of 21h00 and 9h00. Figure 9B: shows the corresponding salivary melatonin levels From Voultsios<sup>50</sup>.

### 3.3 Melatonin levels during pregnancy and postpartum

The changes in serum melatonin concentrations and their roles in pregnancy are not clear<sup>46</sup>. Pregnant women also wake more frequently as the foetus grows. They also often suffer nocturia and difficulty sleeping comfortably as the pregnancy advances<sup>54</sup>. Nutritional, environmental and other factors such as pregnancy can induce the overproduction of superoxide radicals in the cytosol and mitochondria<sup>47</sup>. Oestrogen and progesterone are known modulators of the amplitude and phase of the circadian rhythm<sup>54</sup>. It is possible that changes in these hormones during pregnancy may alter the rhythm in these women, predisposing them to mood alterations as well as sleep disruption<sup>54</sup>. Nakamura et al. investigated changes in serum melatonin concentrations in normal and several pathologic pregnancies in humans with a special focus on pre-eclampsia<sup>46</sup>. Serum melatonin levels have been shown to be suppressed in the first trimester, corresponding with the increase in oxidative stress (**Figure 10A**)<sup>46</sup>. Serum melatonin levels thereafter rise, peaking in the final trimester, and return to baseline postpartum (**Figure 10A**)<sup>46</sup>. An increase in oxidative stress has been suggested to contribute to the pathogenesis of pre eclampsia<sup>46</sup>. In women with severe pre-eclampsia the serum melatonin levels were significantly lower than in healthy controls (**Figure 10B**)<sup>46</sup>. These data suggest that the decrease in melatonin may contribute to the increase in oxidative stress<sup>46</sup>.

Evidence in the literature suggests that melatonin levels return to baseline shortly after birth<sup>46</sup>. Melatonin, as well as circadian sleep rhythm, have conversely been shown to be disturbed in the postpartum period<sup>47</sup>. Postpartum women often wake up more frequently to care for their infant<sup>47</sup>. Stempiank et al. performed a study on the effect of melatonin on the secretion of the nursing hormones oxytocin and prolactin in rats<sup>55</sup>. The study found that concentrations of 1ng/ml of melatonin significantly inhibited the secretion of prolactin in nursing female rats<sup>55</sup>. A similar study performed on ewes, demonstrated that, controlled exogenous melatonin supplementation decreases prolactin secretion<sup>56</sup>. Melatonin may also have additional beneficial effects<sup>46</sup>. Light is a well known trigger for the eclamptic seizure and melatonin is a well known anticonvulsant<sup>46</sup>. Melatonin provided to pregnant women has been shown to protect their foetus from neurological damage<sup>46</sup>.

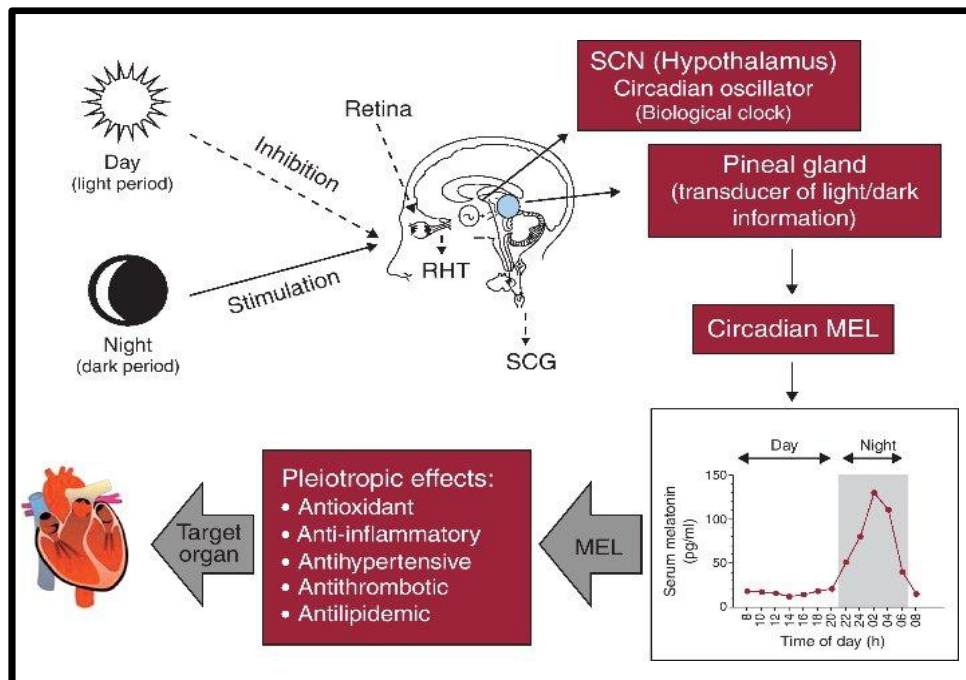


**Figure 10.** Figure 10A: The changes in serum melatonin at night time (solid line) and daytime (dotted line) during a normal singleton pregnancy from conception to postpartum. Figure 10B: the serum melatonin levels in normal pregnancy (white) or pregnancy with mild preeclampsia (hatched) or severe preeclampsia (black). \* $P < 0.01$ , \*\* $P < 0.05$ . From Nakamura et al.<sup>46</sup>

### 3.3 Melatonin and the pathophysiology of cardiovascular disease

Oxidative stress is a strong causative factor in aging and cardiovascular disease<sup>32</sup>. Therapy with antioxidants is a promising treatment in such diseases<sup>32</sup>. Previous studies have, however, failed to show a benefit in using supplementation with classical antioxidant vitamins A, E and C<sup>32</sup>. Melatonin is a multifunctional indolamine which counteracts virtually all pathophysiological conditions and displays significant beneficial actions against peroxynitrite-induced cellular toxicity<sup>32</sup>. Melatonin is also a powerful scavenger of both RNS and ROS, including those formed from peroxynitrite, and blocks transcriptional factors, which induce pro inflammatory cytokines<sup>32</sup>. A preliminary study suggests that melatonin levels are lower in coronary artery disease<sup>45</sup>. Evidence in the literature indicates that melatonin is a promising therapy, either alone or in combination with other antioxidants, in conditions with oxidative stress<sup>32</sup>. Several studies have investigated the effect of melatonin supplementation in atherosclerosis<sup>41</sup>. Preliminary experimental studies in animal models have suggested that melatonin protects the heart and other organs from I/R injury by scavenging the free radicals<sup>41</sup>.

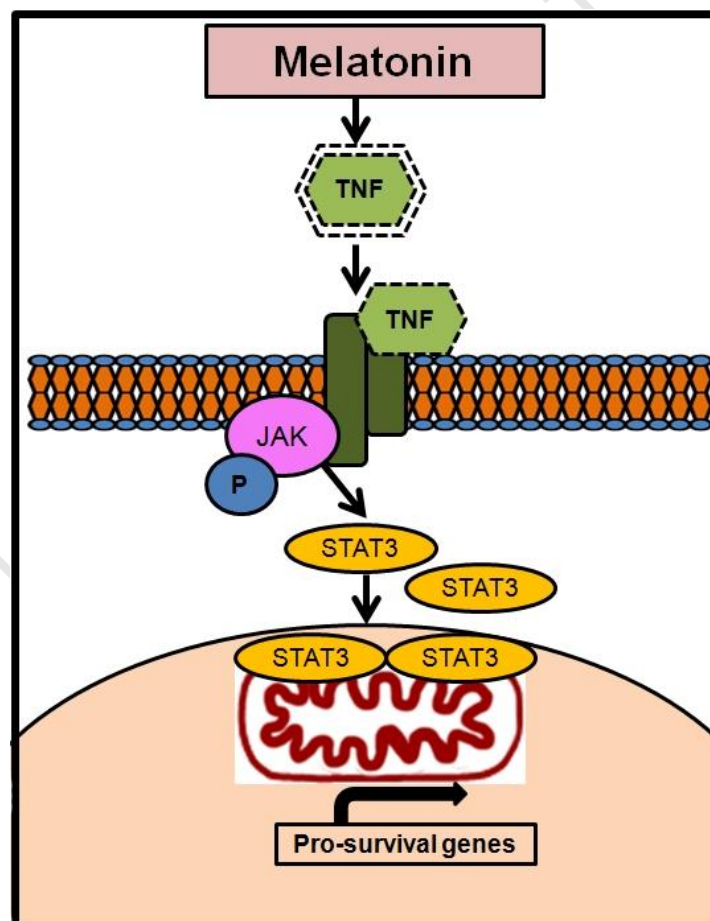
Studies have demonstrated that melatonin has a promising effect on reducing blood pressure (BP)<sup>57</sup>. The hypotensive effect of melatonin is unclear, but may be explained by the free radical scavenging effects or the endothelial relaxing effects of melatonin<sup>57</sup>. Preliminary human trials have demonstrated that, a daily dose of 1mg of melatonin reduced BP in young men and women, compared to placebo supplementation<sup>57</sup>. **Figure 11** demonstrates the physiological regulation of melatonin and its protective effects on the cardiovascular system<sup>58</sup>. In response to light and dark stimuli, melatonin secretion from the pineal gland regulates the melatonin release. This, in turn, has multiple pleiotropic effects on the heart, including anti-inflammatory, antioxidant and antihypertensive properties<sup>58</sup>.



**Figure 11.** Physiological regulation of melatonin by the light/dark environment as detected by the retina. The light dark response and melatonin secretion from the pineal gland regulate the melatonin release. This in turn has multiple pleiotrophic effects on the heart including anti-inflammatory, antioxidant and antihypertensive properties. From Dominguez-rodriguez et al. <sup>58</sup>.

### 3.3.1 Melatonin and inflammation in cardiovascular disease

A study by Lecour's group demonstrated that melatonin activates the powerful cardioprotective survivor activating factor enhancement (SAFE) pathway in male mice<sup>59 60</sup>. Previous animal models have demonstrated that an excess production of inflammatory cytokines, such as TNF $\alpha$  and IL6, contribute to heart failure. However, clinical trials targeting a suppression of inflammatory cytokines in heart failure have failed to translate to humans<sup>59</sup>. Melatonin has been demonstrated to activate the SAFE pathway by activating TNF and TNF receptors on the cell membrane (**Figure 12**)<sup>59</sup>. Once TNF is bound to its receptor, the Janus kinase/signal transducers and activators of transcription 3 (JAK/STAT3) pathway is activated<sup>59</sup>. When activated, JAK proteins are phosphorylated and form a docking site for the activation of STAT3 proteins<sup>59</sup>. The phosphorylation of STAT3 allows homodimerisation and the STAT3 proteins are transported to the mitochondria and induce the transcription of pro-survival genes<sup>59</sup>.



**Figure 12.** Melatonin activates the survivor activating factor enhancement (SAFE) pathway. Melatonin has been shown to activate TNF which binds on its cell surface receptor and subsequently activates the janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) signalling pathway. After phosphorylation, signal transducer and activator of transcription 3 translocates either in the nucleus or in the mitochondria<sup>59 60</sup>.

## 4. SLEEP QUALITY AND CIRCADIAN RHYTHM

### 4.1 Sleep and circadian rhythm

A circadian rhythm is defined as any biological rhythm which displays an endogenous cycle of 24 hours<sup>61</sup>. This rhythm is entrained by external zeitgebers (time givers), in particular, the response to light and dark by the hormone melatonin<sup>62</sup>. The oscillation of the circadian rhythm in humans is controlled by a small group of clock genes within the cell nuclei<sup>62</sup>. These genes are then regulated by a master biological clock in the suprachiasmatic nuclei (SCN)<sup>62</sup>. The SCN gives the precise time cues which regulate a diverse range of physiological, hormonal and behavioural daily patterns<sup>62</sup>. A summary of some of the circadian rhythms in humans is shown in **Figure 13**<sup>45</sup>. Sleep has been characterised by a state of absent or reduced consciousness and the inactivity of all voluntary muscles<sup>63</sup>.

Sleep can be roughly categorised into two main forms, rapid eye movement sleep (REM) and non rapid eye movement sleep (non REM)<sup>63</sup>. The non REM sleep is further divided into 4 stages<sup>63</sup>. Gross brain wave activity during sleep may be measured by the use of an electroencephalogram (EEG). The REM and non REM stages of sleep and the relevant EEG pattern is shown in **Figure 14**. While awake, most people show alpha and beta EEG wave patterns. These beta waves have high frequency, low amplitude, and are desynchronous<sup>64</sup>. During relaxation, the waves begin to slow down and become more synchronous and the EEG amplitude decreases demonstrating more alpha type waves<sup>65</sup>. The first stage of non REM sleep is characterized by theta waves, which are even slower in frequency and greater in amplitude than the alpha waves<sup>65</sup>. As the sleeper moves to stage 2 sleep theta wave activity continues. Stages 1 and 2 are relatively "light" stages of sleep<sup>65</sup>. When individuals are awakened in stages 1 or 2, they often do not realise they have been asleep<sup>65</sup>. Humans pass from the theta waves of stage 1 and 2, to the delta waves of stage 3 and 4. Delta waves are the slowest and the highest amplitude brain waves<sup>65</sup>. Delta sleep is considered as the deepest sleep, the waves being vastly different from the waking brain waves. If an individual wakes up in stages 3 or 4, they are usually sleepy and disoriented<sup>65</sup>. Following the first 4 stages, humans then enter the REM sleep, characterised by rapid eye movement and a dramatic loss of muscle tone. During REM sleep the waves are similar to the waking state and this stage is associated with dreaming<sup>65</sup>. The brain waves then usually either return to an awake state or cycle back to stage 2<sup>65</sup>. A full cycle from stage 1 to REM takes approximately 90 minutes<sup>65</sup>. Throughout the sleep cycle, the delta stages decrease until waking<sup>65</sup>. Any disruption such as frequent waking during the sleep cycle or an insufficient sleep cycle leads to a decline in sleep quality<sup>64</sup>.

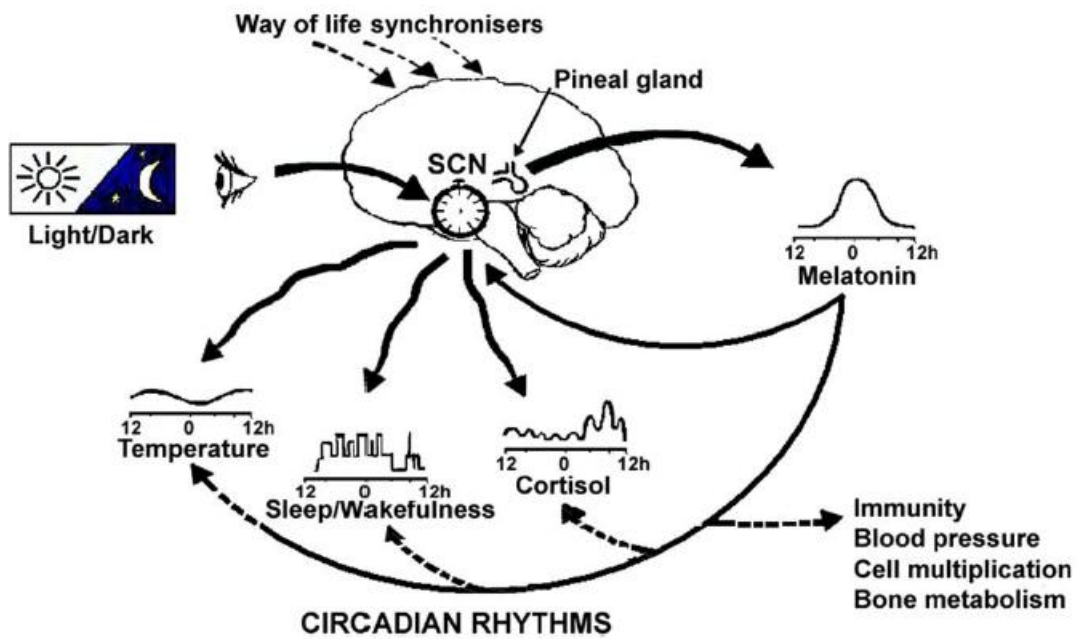


Figure 13. Circadian rhythms are 24 hour cycles including: melatonin, sleep/wakefulness, cortisol and temperature From Claustrat et al.<sup>45</sup>.

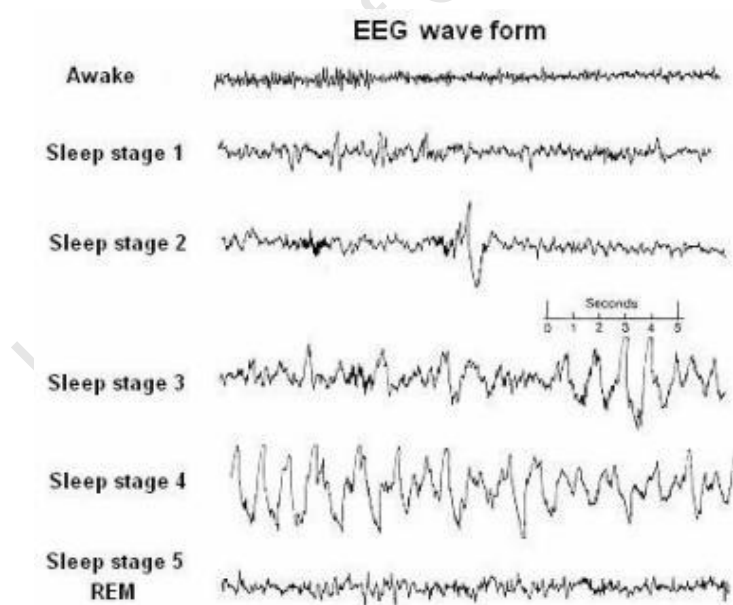


Figure 14. Electroencephalograph (EEG) patterns of wakefulness and the 5 sleep stages. From [www.healthyojas.com](http://www.healthyojas.com)

## 4.2 Sleep quality

Globally, with increasing economic and social demands, the population is rapidly evolving into a 24-hour permanently awake society<sup>66</sup>. Increasingly, humans are working outside of the normal 0800–1700 h working day, with up to 20% of the population working outside of these hours<sup>66</sup>. The negative effects of the change in working hours on sleep loss and, subsequently health, are currently being appreciated<sup>66</sup>. The normal circadian rhythm in individuals with changes in sleeping patterns is disrupted<sup>64</sup>. In humans, sleep is normally initiated by the nocturnal response to dark, accompanied by an increase in melatonin and a decline in temperature<sup>66</sup>. Any change in the normal sleep-wake cycle leads to physiological disturbances, such as melatonin secretion, which has a domino effect on blood pressure, increases in the stress hormone, cortisol, as well as activation of the inflammatory and oxidative stress pathways<sup>66</sup>. Attempts to sleep in an inappropriate phase of the circadian cycle usually result in shorter sleep duration and more nocturnal awakenings<sup>66</sup>. Primary insomnia (difficulty sleeping) is a sleep disorder not caused by organic or psychiatric condition<sup>67</sup>. Insomnia involves a difficulty of initiating, as well as maintaining, sleep<sup>67</sup>. Daytime naps have been shown to increase alertness and sleep efficiency following a disturbed night sleep<sup>68</sup>. Measuring the sleep quantity is an important measure of sleep efficiency<sup>69</sup>. This is however not always directly correlated with the quality of sleep<sup>69</sup>. Nocturnal awakenings are more predictive of sleep quality<sup>69</sup>. Introduced in 1989, the Pittsburgh sleep quality index (PSQI) is a useful tool to measure sleep quality in different patient groups with psychiatric and sleep disorders<sup>67</sup>.

### 4.3 Sleep quality during pregnancy and postpartum

Pregnant women report that during pregnancy their sleep is disrupted<sup>70</sup>. Sleep deprivation has been linked to increases in pro inflammatory cytokine levels<sup>70</sup>. Okun and Coussons-Read. investigated the effect of the altered sleeping patterns on the serum inflammatory cytokines of pregnant women, compared to non pregnant women<sup>70</sup>. The study found that CRP levels, TNF alpha, as well as IL10, significantly correlated with sleep loss during pregnancy<sup>70</sup>. Elevated pro inflammatory cytokines during pregnancy have been linked with conditions such as postpartum depression, pre-eclampsia and PPCM<sup>70</sup>. Sleeping time has been shown to increase during pregnancy, peaking at 33-36 weeks and decreasing again towards delivery.<sup>71</sup>

Although sleep is known to be disrupted in the postpartum period, very few studies have investigated this phenomenon. Thomas et al. investigated the level and timing of melatonin in postpartum and non pregnant women over a 24 hour period<sup>47</sup>. The study found a significantly different pattern of melatonin between the two groups<sup>47</sup>. Postpartum women were found to have a higher baseline and lower maximum melatonin levels, thus suggesting a disruption in circadian rhythm during the postpartum period<sup>47</sup>. Mancini et al. performed a study on the sleep disturbances on non depressed postpartum women<sup>72</sup>. The study found that the total sleep time was not as badly affected as the fragmentation of sleep<sup>72</sup>.

#### 4.4 Sleep and cardiovascular health

Epidemiological studies suggest that circadian disruption contributes to an increased risk in obesity, cardiovascular disease and diabetes<sup>73</sup>. Experimental investigations of sleep deprivation and fragmentation have been found to alter immune responses, as well as increase the circulation of inflammatory markers<sup>74</sup>. Circadian disruption has been shown to increase serum concentration of CRP, an established marker of inflammation and cardiovascular disease<sup>75</sup>. There have been multiple self-reported cases and anecdotal evidence that poor sleep quality and continuity are common in HF patients<sup>76</sup>. A study conducted with post operative cardiac patients indicate that sleep disruption contributes to declines in functional performance, as well as in mental health<sup>77</sup>. The physical and mental effects of sleep disruption have been suggested to be a risk factor, as well as a predictor of poor outcome in cardiac patients<sup>7613</sup>.

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## 5. DEPRESSION

### 5.1 Definition of depression

Depression is a state of low mood which forms part of a set of mood disorders<sup>78</sup>. Mood disorders include major depression, seasonal affective disorder, bipolar illness and postpartum depression<sup>78</sup>. Major depression or unipolar depression is a clinical state of low mood, with loss of interest or pleasure and interferes with daily functioning<sup>79</sup>. Seasonal affective disorder, has a similar presentation to major depression, except with symptoms being more severe or only present in winter months<sup>80</sup>. Bipolar depression involves sometimes rapidly changing mood states from a depressive to a manic state<sup>80</sup>. It has been suggested that up to 20% of all adults will suffer a mood disorder requiring treatment in their life time<sup>78</sup>. There is no currently biological test for clinical depression, a diagnosis is made using psychiatric interview by a qualified psychiatrist<sup>78</sup>. The diagnosis in both clinical practice and research studies by signs and symptoms is presented in **Table 2**. Sleep disturbances, fatigue, lack of concentration and motivation are examples of symptoms of major depression in the diagnostic and statistical manual of mental disorders IV (DSM IV)<sup>78</sup>.

**Table 2:** Diagnostic criteria for major depressive disorder. From Remick et al.<sup>78</sup>

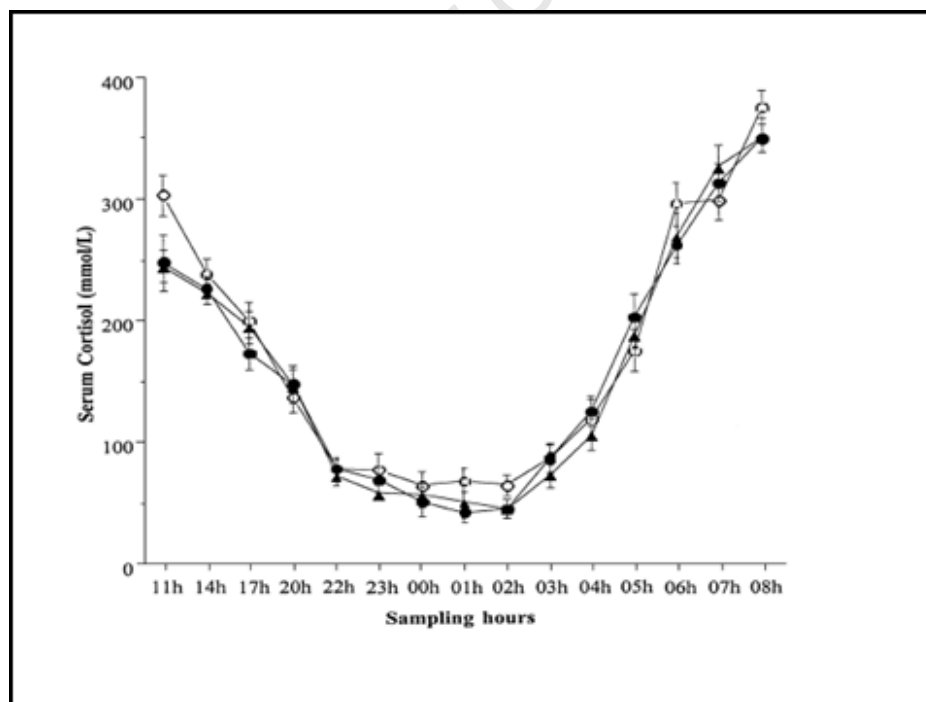
A. The patient has depressed mood (e.g., sad or empty feeling) or loss of interest or pleasure most of the time for 2 or more weeks plus 4 or more of the following symptoms	
Sleep	Insomnia or hypersomnia nearly every day
Interest	Markedly diminished interest or pleasure in nearly all activities most of the time
Guilt	Excessive or inappropriate feelings of guilt or worthlessness most of the time
Energy	Loss of energy or fatigue most of the time
Concentration	Diminished ability to think or concentrate; indecisiveness most of the time
Appetite	Increase or decrease in appetite
Psychomotor	Observed psychomotor agitation/retardation
Suicide	Recurrent thoughts of death/suicidal ideation
B. The symptoms do not meet criteria for a mixed episode (major depressive episode and manic episode)	
C. The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning	
D. The symptoms are not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication) or a general medical condition	
E. The symptoms are not better accounted for by bereavement	
<small>*Adapted from the <i>Diagnostic and Statistical Manual of Mental Disorders</i>, 4th edition.<sup>3</sup></small>	

## 5.2 Depression during pregnancy and postnatal depression

Peripartum depression is a serious and prevalent mental health condition<sup>81</sup>. Depression is disabling for women and is most common during the childbearing years<sup>81</sup>. Postpartum depression refers to the depressions occurring during the postpartum period up until the first year following childbirth<sup>81</sup>. In developed countries studies have shown that the prevalence of postpartum depression is around 10-15%<sup>82</sup>. In developing regions, this percentage is often doubled. A study in Western Nigeria reported the incidence of maternity blues to be 31.3%<sup>83</sup>. A South African study identified that 32% of the perinatal women screened for maternal depression qualified for referral to counselling<sup>84</sup>. This case study claimed that there is a deficiency in screening in the primary health care in South Africa and that many cases are not identified<sup>84</sup>. The study used the Edinburgh Postnatal Depression Scale (EDPS) as a screening tool<sup>84</sup>. The EDPS is a validated 10 item questionnaire used for screening for a probable diagnosis of depression both pre and postpartum<sup>85 82</sup>. In a separate study performed in peri-urban settlements in Cape Town, South Africa investigated the prevalence of depressed mood during pregnancy in this population<sup>85</sup>. The study found that 39% of the pregnant women showed signs of depression. Depression is also a common psychiatric complication resulting from Human immunodeficiency virus (HIV), which is prevalent in South Africa<sup>86</sup>. The symptoms of postpartum depression are similar to those of major depression and they often overlap with anxiety symptoms<sup>81</sup>. The psychosocial risk factors for maternal and postpartum depression include past history of mental illness, mental disturbance during pregnancy, family history of depression, low socioeconomic status and poor interpersonal relationships<sup>87</sup>. Postnatal depression is sometimes precluded by depression during maternity<sup>88</sup>. Bunevicius and colleagues investigated psychosocial risk factors for depression during pregnancy<sup>88</sup>. They determined that low education, psychosocial stressors and previous history of depression were trimester dependant<sup>88</sup>.

### 5.3 Depression and cortisol

Cortisol is a glucocorticoid steroid hormone produced by the zona fasciculata of the adrenal cortex<sup>89</sup>. The hormone is released in response to stress and is commonly elevated in psychiatric conditions such as anxiety and depression<sup>89</sup>. Cortisol levels, like melatonin, follow a circadian rhythm. The normal levels of cortisol therefore vary throughout the day (**Figure 15**)<sup>90</sup>. Previous studies have reported that blood cortisol levels are higher in pregnant woman<sup>91</sup>. In the third trimester, it has been reported that salivary cortisol is twice the values of non pregnant women<sup>91</sup>. Other reports suggest that cortisol can increase up to three-fold during pregnancy<sup>92</sup>. This increase generally returns to baseline shortly after birth<sup>93</sup>. Significantly higher cortisol levels have been reported in postpartum women with psychiatric illness<sup>93</sup>. Goodwin et al. reported an average early morning serum cortisol level in pregnant women of  $30.55 \pm 9.95$  ng/ml<sup>94</sup>. Bhai et al. used the EDPS to measure depression and plasma to measure hormones in pregnant, postpartum and non gravid women. The study found that postpartum women had significantly increased cortisol, prolactin and oestrogen than non gravid women<sup>93</sup>. Depressed postpartum women also had lower levels of prolactin<sup>93</sup>. Women who has breastfed had lower EDPS scores<sup>93</sup>.



**Figure 15.** Circadian rhythms of cortisol in three different days in 10 participants. From Selmanoui and Touitou,<sup>90</sup>

#### 5.4 Depression as a risk factor for cardiovascular disease

Since the times of the ancient Greeks, affective dispositions have been thought to be associated with physical disease<sup>95</sup>. Depression is known to be a risk factor for the development of cardiovascular disease, as well as a predictor of poor prognosis following a cardiac event<sup>96</sup>. Established risk factors such as hypercholesterolemia, hypertension and smoking do not fully correlate to the ischemic heart disease incidence<sup>97</sup>. Psychosocial factors have been suggested to account for these differences<sup>97</sup>. The mental and physiological changes of a depressive individual may also negatively affect the course of the disease<sup>96</sup>. The decrease in the patient's motivation may result in noncompliance with medical recommendations, as well as other factors such as smoking and hypertension<sup>96</sup>. Previous animal and human models have shown links in the pathways between depression and anxiety with physiological responses<sup>95</sup>. Physiologic changes such as nervous system activation, an increase in inflammation, sleep pattern changes and cardiac rhythm disturbances<sup>96</sup>. A recent longitudinal study found a 25 year decrease in life expectancy in individuals with severe mental illness, not from suicide, but from cardiovascular disease<sup>98</sup>.

Inflammatory markers such as CRP, TNF- $\alpha$  and IL6 have been associated with an increased risk for cardiovascular disease<sup>99</sup>. Vaccarino and colleagues investigated the depression, inflammation and cardiovascular outcomes of women<sup>99</sup>. This study found an increased risk of cardiovascular disease was associated with established depression<sup>99</sup>. The study found that women with established depression had 70% higher CRP levels than women without depression<sup>99</sup>. The study found that although established depression was associated with a higher cardiovascular risk, possible depression was not<sup>99</sup>. The study also suggested that the association between depression and cardiovascular disease cannot be explained by inflammation alone<sup>99</sup>. Stewart and colleagues performed a prospective study to determine the directionality of the association between depression and inflammatory markers in both men and women<sup>100</sup>. The study found that only body mass index had a greater association with increased CRP and IL6 than depression<sup>100</sup>.

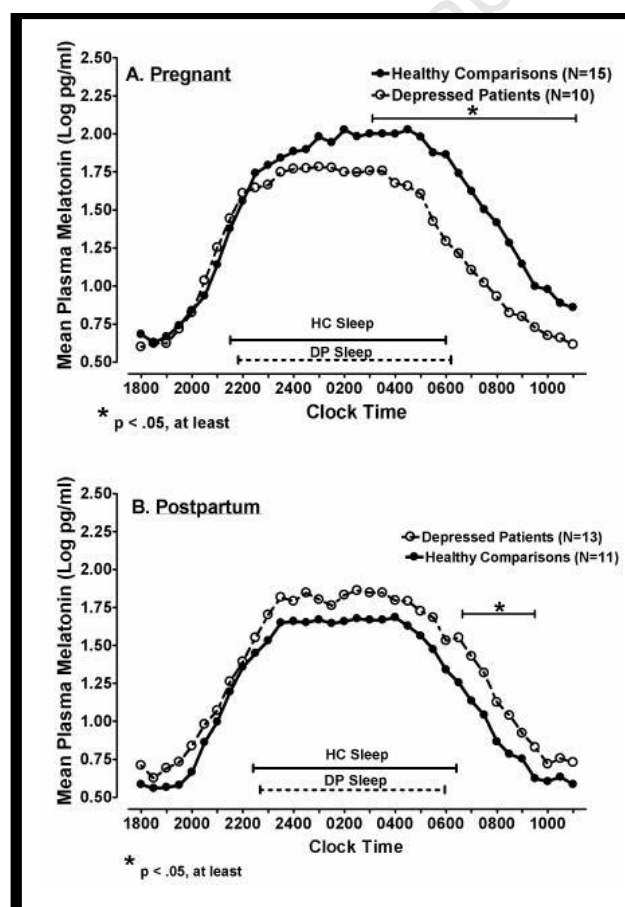
Developing countries have higher frequencies of PPCM, pre-eclampsia and perinatal depression compared to the developed world<sup>101 82</sup>. The mechanism by which depression is thought to contribute is through an increase in oxidative stress and inflammation<sup>99</sup>. Oxidative stress and inflammation have both been suggested to contribute to the development of PPCM and pre-eclampsia<sup>4 102</sup>. Depression and anxiety during pregnancy may contribute to hypertension via excretion of vasoactive hormones or other neuroendocrine transmitters<sup>103</sup>. A prospective population study suggested that depression

and anxiety in early pregnancy was a risk factor for pre-eclampsia later in pregnancy<sup>103</sup>. The oxidative stress and inflammation increased by depression may lead to the development of left ventricular heart failure of PPCM or the hypertension of pre-eclampsia.

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## 5.5 Melatonin and depression

Circadian rhythm disturbance is a major feature of mood disturbances<sup>54</sup>. A study by Taki et al. investigated the relationship between poor sleep quality, mental stress and salivary melatonin concentrations<sup>104</sup>. The study found that subjects with poor sleep quality had, on average, higher nocturnal melatonin values<sup>104</sup>. The study also found individuals with higher anxiety and depression also demonstrated a higher nocturnal melatonin value<sup>104</sup>. This has been suggested to be due to the natural defence response to increase in oxidative stress caused by change in diet or lifestyle in response to mental stress<sup>104</sup>. Chronic poor sleep quality is likely to increase the melatonin concentration in defence against the sleep disruption<sup>104</sup>. Parry et al. measured the levels of plasma melatonin in depressed pregnant and postpartum women<sup>105</sup><sup>54</sup>. The study noted that plasma melatonin levels were lower in pregnant and higher in postpartum depressed women compared to healthy controls (**Figure 16**)<sup>54</sup>. These findings suggest that melatonin levels in depressed women are likely disturbed compared to controls<sup>54</sup>.



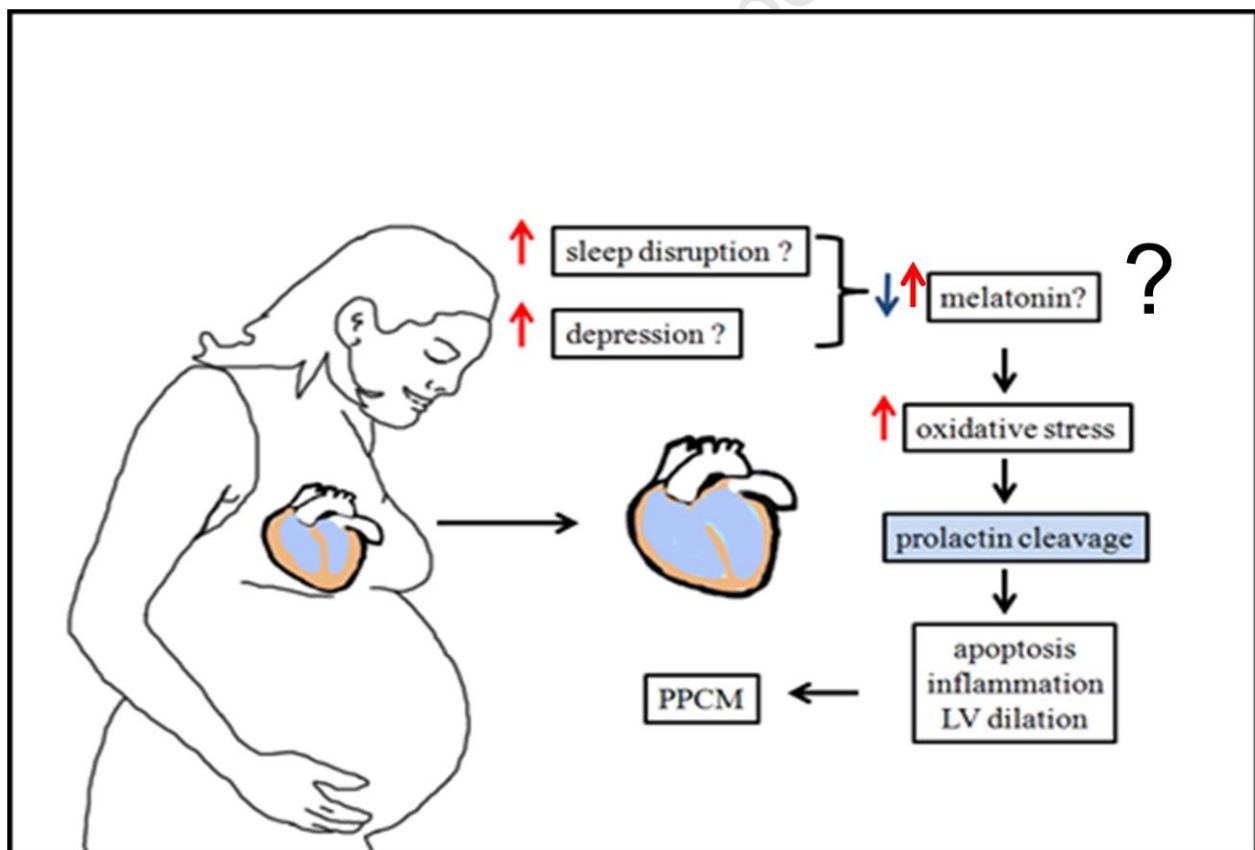
**Figure 16.** Mean plasma melatonin concentrations at successive time points in Healthy Controls (HC; closed circles) women and Depressed Patients (DP; open circles) in (A) Pregnant and (B) postpartum women From Parry et al.<sup>105</sup>

## OBJECTIVES

PPCM is a heart disease of unknown aetiology emerging previously healthy women towards the end of pregnancy or in the first postpartum months. The exact pathophysiology of PPCM remains unclear, however the extensive work of Hilfiker-Kleiner et al. suggests that oxidative stress mediates the cleavage of the nursing hormone prolactin into a 16kDa pro apoptotic form which contributes to PPCM. Melatonin is a powerful endogenous antioxidant that can limit the damaging effect of oxidative stress. Melatonin levels are known to be altered in sleep disruption as well as depression.

**In the present study we hypothesise that, an alteration in the levels of the powerful antioxidant hormone melatonin, may contribute to the pathophysiology of PPCM.**

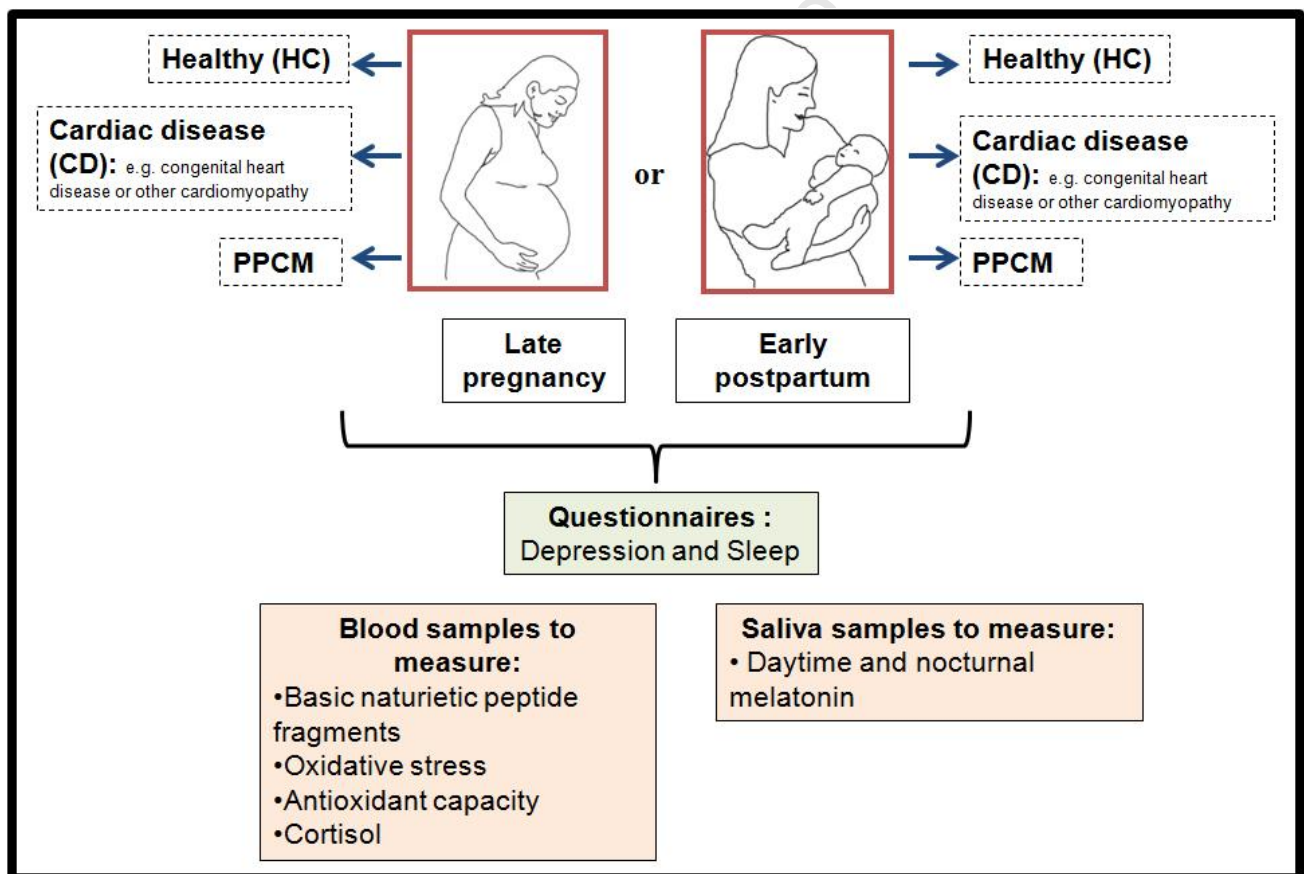
**Figure 17** presents the hypothesis that sleep disruption and depression during pregnancy/postpartum may affect the production of the antioxidant melatonin and increase the oxidative stress, leading to prolactin cleavage and the development of PPCM.



**Figure 17.** This study investigates whether a disruption in the antioxidant melatonin, possibly due to an increase in depression and/or sleep disruption leads to an increase in oxidative stress thereby causing the cleavage of prolactin into its 16kDa pro apoptotic form which has previously been proposed to contribute to the pathogenesis of PPCM.

To explore this hypothesis, patients with or without PPCM were recruited from the cardiac disease in maternity clinic (CDMC), Groote Schuur Hospital (GSH), Cape Town South Africa as well as the general maternity clinic and the following experiments were performed (**Figure 18**):

1. Questionnaires were administered to the patients to determine the self reported depression and sleeping pattern disruptions.
2. Blood samples were taken from the patients to measure markers of oxidative stress and antioxidant capacity. Cortisol levels were assayed to determine the levels of the stress hormone in the patients. A BNP fragment assay was used as a measure of the severity of heart failure.
3. Saliva samples from the patients were taken for measurement the baseline and nocturnal melatonin levels.



**Figure 18.** Summary of patient recruitment and sample collection. Late pregnant or early postpartum women with PPCM, other cardiac disease (CD) or healthy women (HC) were recruited for the study. The women were given questionnaires and blood and saliva samples were taken for further biochemical assays.

## CHAPTER 2: MATERIALS AND METHODS

### 2.1 Patient recruitment and sample collection

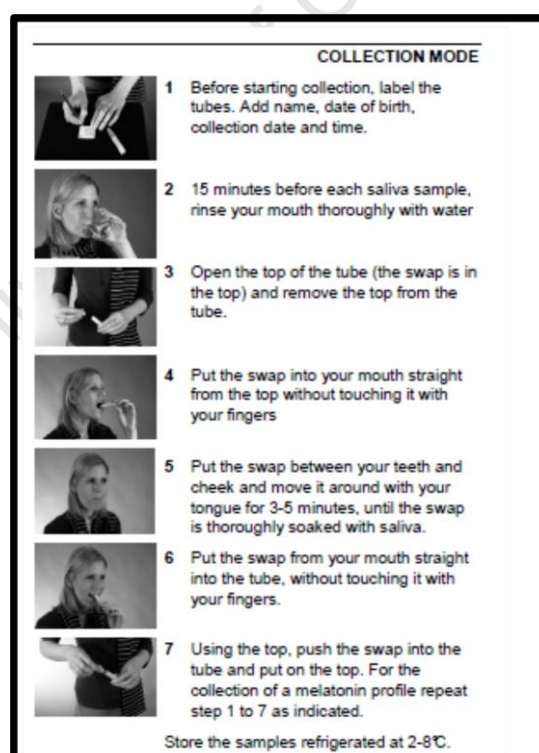
Pregnant (third trimester) and postpartum women of childbearing age (18-45) were recruited for the study (**Figure 19**). Women with PPCM on stable therapy were the experimental group. The majority of patients were diagnosed in the first months postpartum, even though the initial signs and symptoms had been present towards the end of pregnancy<sup>11</sup>. The pregnant PPCM group are women, previously diagnosed with PPCM, who are at risk for reoccurrence. Women with other pre-existing cardiovascular disease in maternity (CD), such as cardiomyopathy (CM) or congenital heart disease (CHD), were also recruited. The PPCM and CD groups were recruited from the CDMC at Groote Schuur hospital (GSH), Cape Town, South Africa. Healthy control (HC) women, without any known cardiovascular disease, were recruited from the maternity ward at GSH. A total of 24 HC (12 postpartum and 12 pregnant), 40 CD (23 pregnant and 17 postpartum) and 14 PPCM women (5 pregnant and 9 postpartum) were recruited (**Table 3**). This project is an extension of the research activities in the CMDC at Groote Schuur Hospital (HREC/ref:176:2010). The study has been approved by the UCT Human Ethics Research Committee (HREC/ref:246:2012).

**Table 3.** Number of pregnant and postpartum HC, CD and PPCM patients recruited for the study

	<u>HC</u>	<u>CD</u>	<u>PPCM</u>
<b>Pregnant</b>	12	23	5
<b>Postpartum</b>	12	17	9
<b>Total</b>	24	40	14

The women were questioned using two separate questionnaires to measure their depression risk, as well as the quality of their sleeping patterns. Two tubes of blood (one coated with ethylenediaminetetraacetic acid (EDTA) to collect plasma and one without for serum collection) was collected from each patient, between 10 and 11am, and taken back to the lab for processing. The tubes were spun down for 5 minutes at 2200 rpm at 4°C. The samples were then stored at -80°C until analysis.

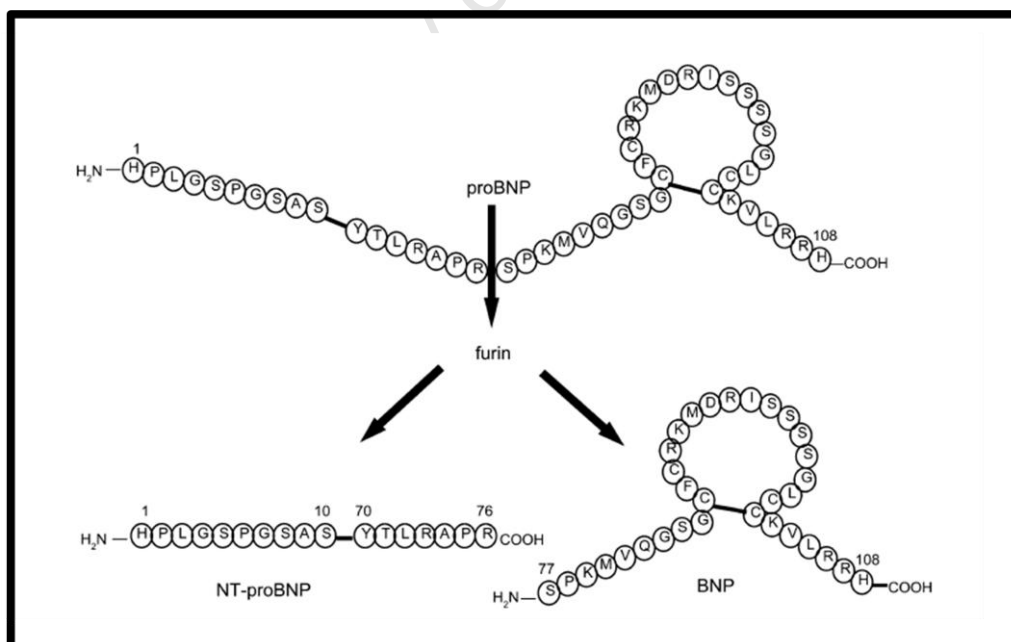
For the collection of saliva, each patient was given 2 saliva collection tubes (Salivette®, Sardstedt, Germany) and a set of pictorial (**Appendix A**) and written instructions according to manufacturers guidelines (**Figure 19**). The patients were instructed to avoid bright light in favour of muted light. The patients were also instructed not to consume any food 30 minutes prior to the collection of the samples and to avoid eating bananas and chocolate for the entire day before. They were also instructed not to consume caffeinated beverages or alcohol on the evening of collection. The patients were instructed to provide baseline samples at 12pm and nocturnal samples at 2am. The tubes were then collected from the patients, processed and stored at -80°C until analysis.



**Figure 19.** Patient saliva collection instructions provided for each patient. The patients were each supplied with 2 tubes for samples at time points 12pm (baseline) and 2am. The patients were instructed to refrigerate the samples and return them the following day (Appendix A).

## 2.2 Basic natriuretic peptide (BNP) fragment enzyme immunoassay

The measurement of B-type natriuretic peptide (BNP) concentrations in blood is a useful marker of heart failure<sup>12</sup>. The BNP hormone is part of a family of structurally similar hormones, including atrial natriuretic peptide (ANP), C-type natriuretic peptide (CNP), and urodilatin<sup>12</sup>. The BNP and ANP are secreted by ventricular myocytes, although BNP is mainly secreted from the left ventricle and is the most commonly used as a marker of heart failure<sup>12</sup>. BNP preferentially binds to a membrane-bound guanylyl cyclase (GC) receptor. The peptide plays an important role in the regulation of cardiovascular and renal homeostasis, as well as the regulation of fatty acid metabolism and body weight. The major precursor protein of BNP is proBNP, which is cleaved to form BNP and biologically inactive NT-proBNP (**Figure 20**)<sup>12 106</sup>. There remains a debate when and where the pre hormone is split. Data from in vitro experiments suggests the proteolytic enzyme, furin is responsible for the cleavage<sup>107</sup>. This cleavage, releases active BNP which leads to natriuresis, diuresis, vasodilatation and smooth muscle relaxation<sup>12</sup>. ANP performs similar functions to BNP, but is produced by the atria<sup>12</sup>. The serum concentrations of serum BNP rise in pathological states, such as in ventricular dilation or reduced clearance of peptides (renal failure)<sup>12</sup>. Studies have shown the greater cardiac damage, the greater the BNP concentration<sup>12</sup>.

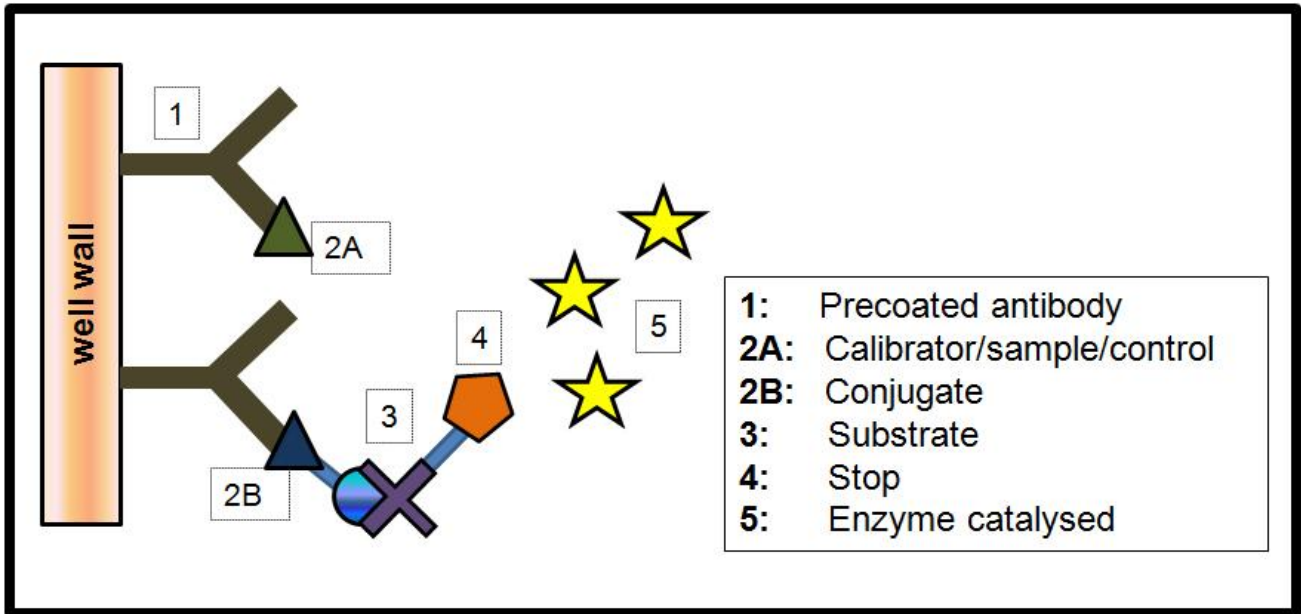


**Figure 20.** Schematic drawing of proBNP showing enzymatic cleavage into biologically active fragments, NT-proBNP and BNP

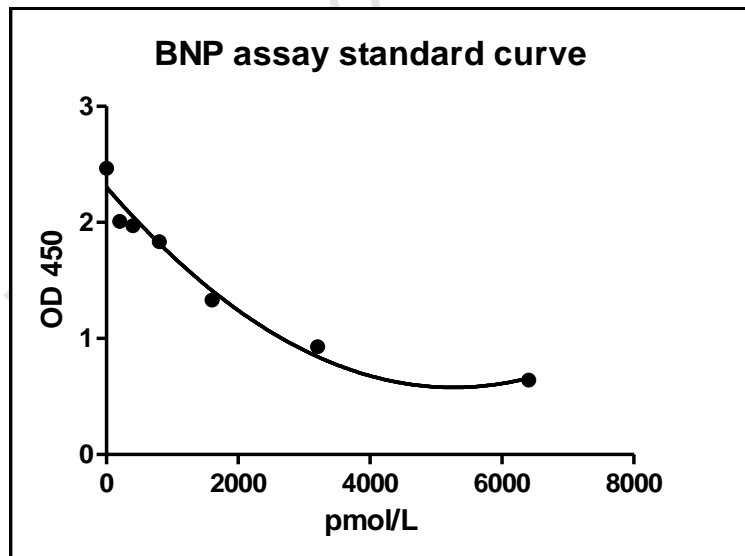
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The BNP fragment levels in the serum of all patients, were quantified using a BNP fragment assay as per manufacturer's instructions (Biomedica, Netherlands, BI-20852W). BNP fragments in the serum are very heterogeneous. Mature BNP consists of 108 amino acids (proBNP and BNP), and undergoes cleavage resulting in the function of the physiologically active BNP-32, as well as physiologically inactive N-terminal peptide comprising amino acids 1-76, which is further degraded proteolytically.

The serum samples and kit reagents were defrosted on ice, then acclimatised to room temperature (RT). 150µl assay buffer was aliquoted into each well of a microtiter plate, followed by 30µl of the standards. Positive controls and human samples were added into the wells in duplicate. The BNP fragments in the serum samples bind to the antibodies pre-coated on the plate (**Figure 20**). 50µl of the conjugate was then added and the plate was swirled gently, to coat the unbound antibodies. The plate was then sealed and incubated overnight in the dark. The following day, the liquid was aspirated and the wells were washed 5 times with 300µl of the wash buffer. The substrate (200µl) was added into each well to bind to the conjugate. The plate was then incubated for 20 minutes at RT in the dark. The stop solution (50µl) was added and the absorbance was measured at 450nm, with a reference at 630nm (Spectramax plus384 fluorescence spectrophotometer (Abotech, South Africa) using Softmax pro software (version 4.4)). The absorbance measured is inversely proportional to the BNP concentration. A standard curve was plotted and the BNP concentration expressed in pmol/L of the samples was calculated by extrapolating the values from the standard curve (**Figure 22**). The serum BNP concentration was converted to pg/mL using the manufacturer recommended conversion factor of 8.475. According to the European Society of Cardiology (ECS) Guidelines in 2012 the optimal upper cut-off/exclusion point for healthy BNP is 100pg/mL<sup>108</sup>.



**Figure 21.** The principle of the BNP fragment assay. The wells of the commercial microtiter plate are coated with BNP specific antibodies. Either BNP from the standards or samples binds to the antibodies. The conjugate binds to the remaining antibodies. The substrate binds to the conjugate and the relative fluorescence is measured at an optical density (OD) of 450nm. The intensity of the fluorescence of the conjugate/substrate reaction is inversely proportional to BNP concentration of the samples.

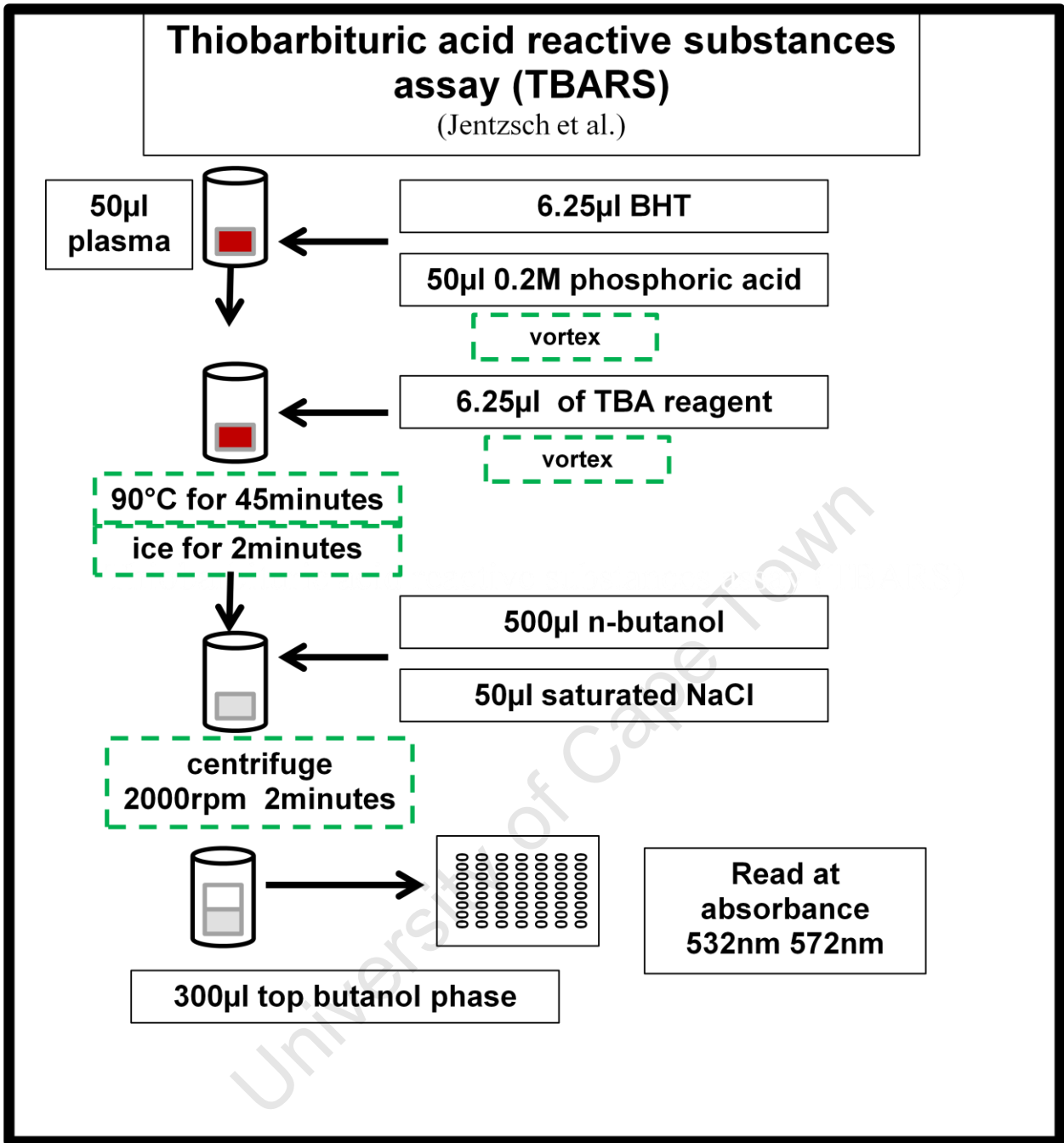


**Figure 22.** Basic natriuretic peptide (BNP) fragment assay standard curve generated from samples with known concentrations of BNP. The intensity at optical density of 450nm is inversely proportional to the BNP concentration of the known standards in pmol/L.

### 2.3 Thiobarbituric acid reactive substances assay (TBARS)

Oxidative stress has been suggested to potentially contribute to the pathogenesis of PPCM<sup>18</sup>. The direct measurement of ROS is challenging because of their very short half lives<sup>109</sup>. It is therefore preferable to measure the damaged by-products, rather than the ROS themselves<sup>109</sup>. The thiobarbituric acid reactive substances (TBARS) are formed subsequent to lipid peroxidation and may be detected using thiobarbituric acid (TBA) as a reagent<sup>109</sup>. The assay measures malondialdehyde (MDA), a low molecular weight end product of lipid peroxidation<sup>109</sup>.

To measure the MDA in the plasma of all patients, a TBARS assay was performed according to the method of Jentsch et al.<sup>110</sup>. A schematic diagram of the method used is shown in **Figure 23**. Initially, 50µl of plasma was aliquoted in duplicate into 1.5ml Eppendorf® tubes and mixed with 6.25 µl of butylated hydroxytoluene (BHT/C<sub>2</sub>H<sub>5</sub>OH)(Fluka-Chemie, Buchs, Switzerland) in ethanol (Merck Chemicals, Cape Town, South Africa) as well as 50µl 0.2M phosphoric acid (Merck Chemicals, Cape Town, South Africa). The tubes were then vortexed. 6.25µl of 0.1M TBA reagent in 0.1M NaOH) (Sigma-Aldrich, Cape Town, South Africa) was added and the tubes were vortexed again. The tubes were incubated at 90°C for 45 minutes on a heating block and the reaction was stopped by cooling the tubes in ice for 2 minutes. MDA was extracted with 500µl n-butanol and 50µl saturated NaCl to facilitate phase separation. The mixture was centrifuged at 2000rpm for 2 minutes. 300 µl of the top butanol phase of samples were aliquoted into a flat bottomed 96 well microtiter plate. The absorbance was read at an OD of 532nm 572nm (reference range) on a Spectramax plus384 fluorescence spectrophotometer (Abotech, South Africa), using Softmax pro software (version 4.4), and values were expressed in µmol of MDA per litre of plasma. The Beer-Lamberts law was used to calculate the concentration of MDA in the samples with an extinction coefficient of  $1.54 \times 10^5 \text{ M}^{-1}/\text{cm}^{-1}$ . The average value for healthy subjects reported by Jentsch et al. was  $(0.47 \pm 0.12 \text{ µmol/L MDA})$ <sup>110</sup>

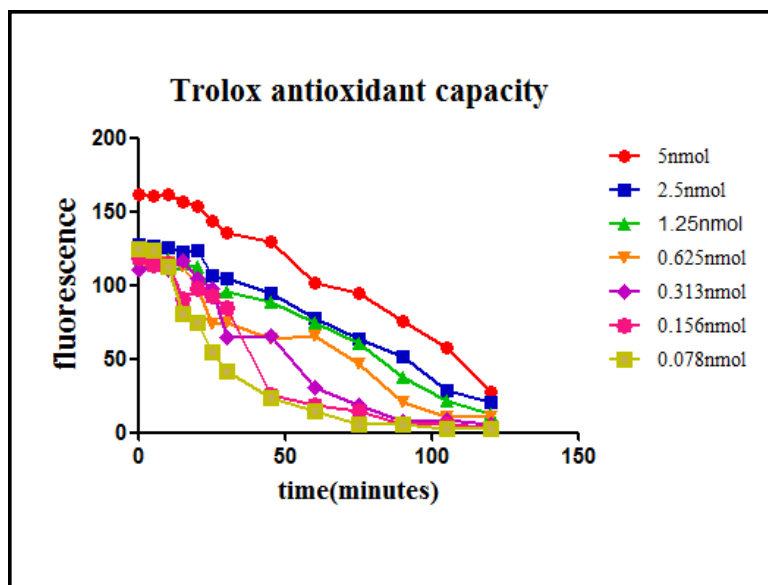


**Figure 23.** Schematic diagram of method of Thiobarbituric acid reactive substances assay (TBARS).

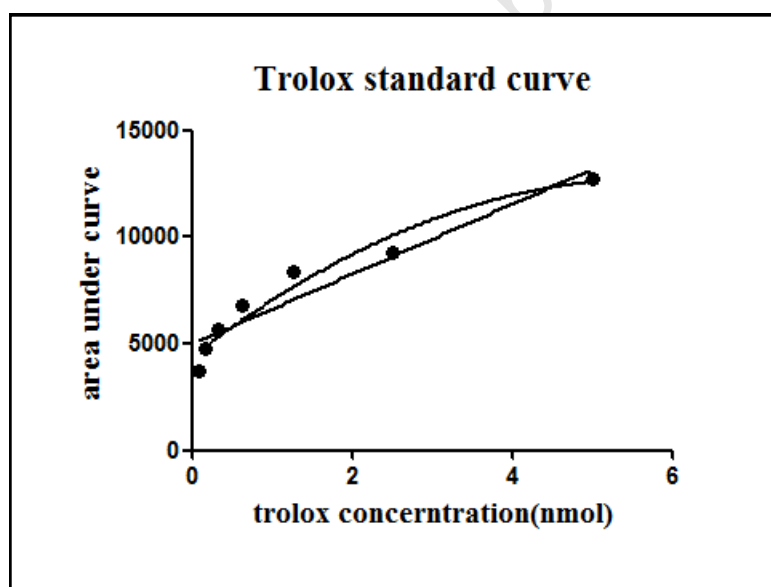
## 2.4 Oxygen radical absorbance capacity (ORAC)

The shift in the oxidative balance occurs when the production of free radicals exceeds the antioxidant capacity (pathways which provide protection against the harmful effects of free radicals)<sup>33</sup>. The total antioxidant capacity of the plasma (of all patients) was assessed using the oxygen radical absorbance capacity (ORAC) assay. This assay evaluates the samples intrinsic antioxidant protective effect of a plasma sample against oxidative degradation of a fluorescent molecule fluorescein (3, 6, dihydroxyspiro(isoberyofuran-1(3H),9(9H)-xanthen)(disodium)(Sigma-Aldrich,Germany), following mixing with 2,2'-azobis (2-amidinopropane)dihydrochloride (AAPH), a peroxy radical generator<sup>111</sup>. The fluorescent intensity of the fluorescein decreases as the oxidative damage of the free radicals released by AAPH progresses. The protection against the free radicals generated by AAPH of the plasma is compared to known concentrations of the vitamin E analogue trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Sigma-Aldrich, Germany)..

A 0.075M pH 7.4 phosphate buffer was prepared and used in all dilutions (**Appendix B**). Different concentrations of trolox were also prepared (**Appendix B**). Working stock of fluorescein was prepared from stock solutions (**Appendix B**). To extract the protein from the plasma, 200µl ice cold ethanol was added to 100µl of plasma. The mixture was then centrifuged at 12000 rpm at 4°C for 5 minutes. The protein free supernatant was then diluted 363x in the phosphate buffer. The trolox standards of known concentrations and plasma samples were aliquoted onto a white flat-bottomed microtiter plate with the phosphate buffer and fluorescein. Warm 0.032M of AAPH was then added to each well and the samples were read over a period of 2 hours at an excitation wavelength of 425nm and an emission wavelength of 520nm on a Varian Cary eclipse fluorescence spectrophotometer (Set point tech., South Africa). The decay curve of intensity of fluorescence vs time was recorded of the samples and trolox standards recorded. The area under the decay curve over 2 hours of each known concentration of trolox was calculated (**Figure 24**). A standard curve was generated using the known concentrations of trolox, compared to the area under the curve (**Figure 25**). The area under the curve is inversely proportional to the antioxidant capacity as a small area indicates faster decay and less protection against the free radicals generated by AAPH. The area under the decay curve over time of the patient plasma samples was calculated and the concentration of trolox equivalents of antioxidant capacity in nmol was extrapolated from the trolox standard curve.



**Figure 24.** Decrease in fluorescence over time in difference of different concentrations of the vitamin E analogue trolox. The area under each curve was for each concentration trolox in nmol was used to generate a standard curve.



**Figure 25.** Standard curve of trolox concentration vs area under curve from figure 24.

## 2.5 Questionnaire selection and design

A questionnaire is an important tool to gather information in the medical field<sup>112</sup>. The design of a questionnaire, such as attention to flow, format and length is of importance to ensure that the questionnaire establishes the correct information.<sup>112</sup>. The first recommended step in the decision to use a questionnaire is to perform a literature search and investigate what type of questionnaires have been previously used<sup>112</sup>. Previously used questionnaires have already been validated and are preferable. Only when no appropriate questionnaire is available should a new questionnaire be designed as this is a tedious process<sup>112</sup>. When designing a questionnaire, the appropriateness of the content, level of language sophistication, sequence and how data is sought from the respondents (self administered or investigator administered) need to be taken into consideration.

The validity of a questionnaire is the degree to which the assessment measures what it is designed to measure. There are three types of validity i) content validity, ii) criterion- related validity, and iii) construct validity. A questionnaire must undergo a validation procedure to ensure that it will accurately achieve its goal. A valid questionnaire must have the following characteristics (i) simplicity and viability (ii) reliability and precision in the words (iii) adequacy for the problem intended to measure (iv) reflect underlying theory or concept to be measured and (v) capacity to measure change<sup>112</sup>. The types of questions are also important. Open ended questions allow the participant to answer in a broad range. Closed ended questions require the participant to answer from a predefined set<sup>112</sup>. To quantify possibly depression, the validated EDPS scale was used directly, as it has previously been validated for use in the South African population<sup>112</sup>. To measure the differences in sleep quality, a sleep quality questionnaire was adapted from the PSQI.

### 2.5.1 The Edinburgh postnatal depression scale

To screen for possible depression the EDPS was administered to all patients (**Figure 26**). The EDPS scale has previously been validated in South Africa<sup>84</sup>. The scale is an efficient and reliable screening tool for perinatal depression. The questionnaire consists of 10 items and is scored on a scale of 0-3. The participants were instructed to answer the questionnaire relating to how they were feeling the previous week. Each item consists of a statement and four possible answers. The minimum score is 0 and the maximum score is 30. A score above 10 warranted further investigation and was considered as possible depression.

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In the past 7 days:

1. I have been able to laugh and see the funny side of things
  - As much as I always could
  - Not quite so much now
  - Definitely not so much now
  - Not at all
2. I have looked forward with enjoyment to things
  - As much as I ever did
  - Rather less than I used to
  - Definitely less than I used to
  - Hardly at all
- \*3. I have blamed myself unnecessarily when things went wrong
  - Yes, most of the time
  - Yes, some of the time
  - Not very often
  - No, never
4. I have been anxious or worried for no good reason
  - No, not at all
  - Hardly ever
  - Yes, sometimes
  - Yes, very often
- \*5. I have felt scared or panicky for no very good reason
  - Yes, quite a lot
  - Yes, sometimes
  - No, not much
  - No, not at all
- \*6. Things have been getting on top of me
  - Yes, most of the time I haven't been able to cope at all
  - Yes, sometimes I haven't been coping as well as usual
  - No, most of the time I have coped quite well
  - No, I have been coping as well as ever
- \*7. I have been so unhappy that I have had difficulty sleeping
  - Yes, most of the time
  - Yes, sometimes
  - Not very often
  - No, not at all
- \*8. I have felt sad or miserable
  - Yes, most of the time
  - Yes, quite often
  - Not very often
  - No, not at all
- \*9. I have been so unhappy that I have been crying
  - Yes, most of the time
  - Yes, quite often
  - Only occasionally
  - No, never
- \*10. The thought of harming myself has occurred to me
  - Yes, quite often
  - Sometimes
  - Hardly ever
  - Never

### SCORING

#### QUESTIONS 1, 2, & 4 (without an \*)

Are scored 0, 1, 2 or 3 with top box scored as 0 and the bottom box scored as 3.

#### QUESTIONS 3, 5-10 (marked with an \*)

Are reverse scored, with the top box scored as a 3 and the bottom box scored as 0.

Maximum score: 30  
Possible Depression: 10 or greater  
Always look at item 10 (suicidal thoughts)

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Figure 26. Edinburgh postnatal depression scale (EDPS) to determine possible depression (Appendix C).

### 2.5.2 Sleep quality questionnaire

Introduced in 1989, the Pittsburgh Sleep Quality Index (PSQI) has been accepted as a useful tool in different patient groups to assess sleep quality in different somatic conditions<sup>67</sup>. The PSQI was not designed to assess the quality of sleep before, during and after pregnancy. We therefore had to adapt the questionnaire, so that it would be more applicable for pregnant and postnatal patients. The sleep quality questionnaire that we adapted, was designed to assess the self-reported sleep quality 6 months prior to pregnancy (baseline), the final month of pregnancy and the first month postpartum. The pregnant and postpartum patients, were all were asked the same questions during the interview, to recall their current and previous sleeping patterns.

#### Questions 1-3: Time of onset of sleep and waking

The first question asked the participant what time they went to bed at night. A Likert scale of 5 options were given (**Figure 27**). The optimum time for circadian rhythm is between 8pm and 10pm. Going to bed before 6pm or after 12pm is considered as an abnormal sleeping rhythm.

The second question asked how long the patient takes to fall asleep at night. Taking long to fall asleep (more than 30 minutes to an hour) indicates the participant has some difficulties falling asleep.

The third question asked what time the participant woke up in the morning. Waking up abnormally early, (before 12pm or between 1 and 3 am) or abnormally late (after 8am) indicates a disruption in circadian rhythm.

#### Questions 4-6: Sleep disruption

The fourth question asked how many times the participant woke up at night. A score of 0 was given if they never woke up, and a score of 5 was given if they woke more than 5 times. The fifth question asked if the patient did wake up, how long they were awake for. This determined whether they had the opportunity to complete the sleep cycle. Waking multiple times during the night for long duration indicates the participant has a more disturbed sleeping pattern. Question 6 assessed how many hours of sleep the participant got at a time between waking. Getting short amounts of sleep between waking indicates a more disturbed sleeping pattern.

### **Questions 7-8: Daytime napping**

Evidence in the literature suggests that individuals who take day time naps after a disturbed night's sleep have beneficial effects on their quality of life. Question 7 asked the participant if they took daytime naps and question 8 asked how long does the naps lasted for? This determined whether the participant benefitted from additional sleep during the daytime hours. A score of 1 was given if the participant took daytime naps and their nap time was scored from 1 for (less than 10 minutes) with a maximum score of 5 if their daytime naps were longer than 5 hours. A score of 2 was given for question 7 if they did not report taking daytime naps.

### **Questions 9-10: Sleep efficiency and ease to awake**

Question 9 assessed whether the participant felt fatigued, rested or very well rested following sleeping. This was to determine whether their sleep was efficient if they felt rested, or if their sleep was disturbed or low quality with them still feeling fatigued on waking. A Likert scale of options was given ranging from fatigued (a score of 1), to very well rested ( a score of 5). Question 10 assessed how easy it was for the participant to wake following sleeping to assess whether they were light or heavy sleepers. A score of 1 was given if they were easily awakened, to 5 if they found it very difficult to wake.

### **Question 11: Caffeine intake**

Question 11 asked the participant how much caffeine they took in units (cups) per day of coke or coffee. Caffeine has been shown to decrease melatonin levels and this question investigated whether the participants drank excessive caffeine or more than 3 units per day. A score of 0 was given if the participants reported that they never consumed caffeine, a score of 1 was given if the consumed 1 unit of caffeine per day, a score of 2 for 2 units, 3 for units and a score of 4 for more than 4 units.

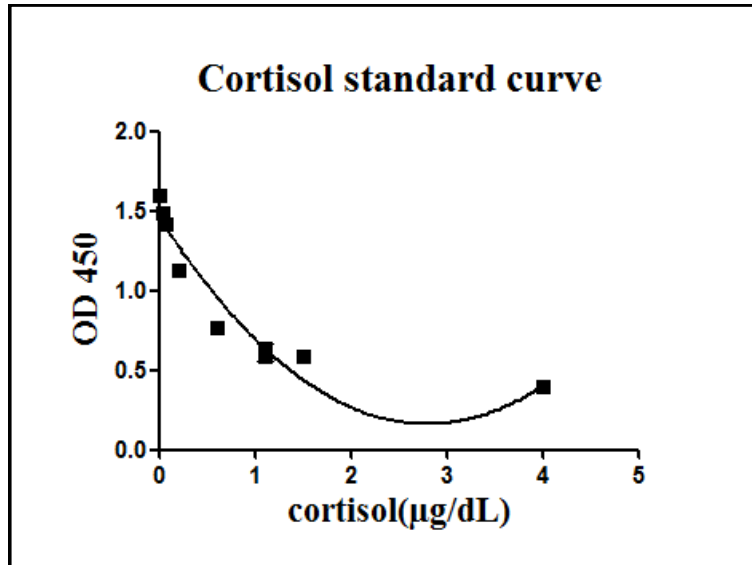
Question	6 months prior to pregnancy (retrospective)					Final month of pregnancy					1 month postpartum				
1.) What time do you usually go to bed at night?	<6pm	6-8pm	8-10pm	10-12pm	<12pm	<6pm	6-8pm	8-10pm	10-12pm	<12pm	<6pm	6-8pm	8-10pm	10-12pm	> 12pm
2.) How long does it take you to fall asleep at night?	<10 min	30 min	1hour	2 hours	>3 hours	<10 min	30 min	1hour	2 hours	>3 hours	<10 min	30 min	1hour	2 hours	>3 hours
3.) What time do you wake up in the morning?	<12pm	1-3am	4-6am	6-8am	> 8am	<12pm	1-3am	4-6am	6-8am	> 8am	<12pm	1-3am	4-6am	6-8am	> 8am
4.)How often do you wake up at night?	never	1-2	2-3	3-4	>5 times	never	1-2	2-3	3-4	>5 times	never	1-2	2-3	3-4	>5 times
5.)When you wake at night how long are you awake?	<10min	10-30min	30-60min	1-2rs	>3hours	<10min	10-30min	30-60min	1-2rs	>3hours	<10min	10-30min	30-60min	1-2rs	>3hours
6.)How many hours sleep do you get at a time?	<1 hr	1-2 hrs	2-4hrs	4-6hrs	>8 hrs	<1 hr	1-2 hrs	2-4hrs	4-6hrs	>8 hrs	<1 hr	1-2 hrs	2-4hrs	4-6hrs	>8 hrs
7.)Do you nap during the day?	yes	no				yes	no				yes	no			
8.) If yes to q7, how long	<10 min	30 min	1hour	2 hours	>3 hours	<10 min	30 min	1hour	2 hours	>3 hours	<10 min	30 min	1hour	2 hours	>3 hours
9.)Do you feel rested following sleep/nap?	fatigued	-fatigued	rested	+rested	++rested	fatigued	-fatigued	rested	+rested	++rested	fatigued	-fatigued	rested	+rested	++rested
10.) How easily are you awakened from sleep	easily	-easily	average	difficult	+difficult	easily	-easily	average	difficult	+difficult	easily	-easily	average	difficult	+difficult
11.)How often do you drink coke/coffee	never	1/day	2/day	3/day	>4/day	never	1/day	2/day	3/day	>4/day	never	1/day	2/day	3/day	>4/day

**Figure 27.** Sleep quality questionnaire comparing sleep quality 6 months before pregnancy, in the final month of pregnancy and the first month postpartum (Appendix D)

## 2.6 Cortisol quantification by Enzyme-linked immunosorbent assay (ELISA)

Cortisol levels in the serum of the HC, CD and PPCM groups were measured using an ELISA assay for human serum or saliva (Immuno-Biological Laboratories, USA) (lot no ECO127). The assay measures active free cortisol in saliva or (hydrocortisone and hydroxycorticosterone). Cortisol is a hormone which is vital for several functions in the human body<sup>89</sup>. These functions include the regulation of blood pressure, cardiovascular function and the metabolism of carbohydrates, proteins and fats. The secretion of cortisol is increased in response to stressors such as trauma, illness or psychological stressors<sup>89</sup>.

The IBL - America Salivary Cortisol HS ELISA Kit is a solid phase ELISA, based on the principle of competitive binding. The serum samples were diluted (1:50) and 50µl of the diluted samples, controls and calibrators were loaded onto a 96 microtiter plate in duplicate. The plate is coated with a polyclonal rabbit antibody directed towards an antigenic site on the cortisol molecule. A 100 µl of enzyme conjugate was aliquoted into each well and the plate was incubated at RT on an orbital shaker at (400-600rpm) for 1 minute and then for 120 minutes at RT (18-25°C). The endogenous cortisol of the patient samples competes with a cortisol-horseradish peroxidase conjugate for binding to the coated antibody. Following incubation the unbound conjugate was washed using wash buffer. Substrate solution (100 µl) was then added into each well and the plate was incubated at RT on the orbital shaker (400-600rpm) for 30 minutes. The amount of bound peroxidase conjugate is inversely proportional to the concentration of cortisol in the sample. After addition of the substrate solution, the intensity of colour developed is inversely proportional to the concentration of cortisol in the patient sample. A 100 µl of stop solution was then added to each well and the colour changed from blue to yellow. The plate was read on a photometer at 450nm and 650nm (reference range) (Spectramax plus384 fluorescence spectrophotometer (Abotech, South Africa) within 15 minutes of the addition of the stop solution. A standard curve was generated using the standards provided in the kit and the cortisol concentration was calculated using the curve as reference (**Figure 28**). The reportable range of cortisol in serum is 0.75-200µg/dL cortisol.



**Figure 28.** Standard curve of the cortisol of known concentrations (x axis) and the corresponding OD 450 absorbance values

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## 2.7 Melatonin quantification by Enzyme-linked immunosorbent assay (ELISA)

Melatonin is a powerful natural antioxidant and acts as endogenous free radical scavenger and broad spectrum antioxidant<sup>47</sup>. Melatonin a major regulator of the circadian rhythm of sleep timing and onset, is modified during physiological states such as pregnancy and may be altered in cardiovascular disease such as high blood pressure<sup>46</sup>.

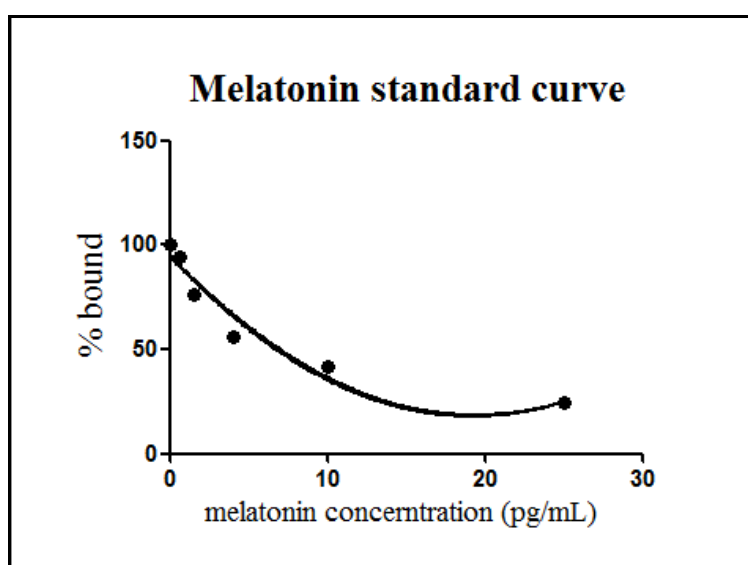
The Bülmann direct saliva melatonin ELISA (batch:21.EK.DSM) kit was used to quantitatively determine the melatonin content in the saliva of the patients. Saliva collection has advantages over plasma and urinary measurements. First, it is more acceptable for participants as it is not painful or invasive. Secondly, melatonin secretion peaks at night and patients can be trained to collect the samples themselves. Melatonin concentrations in saliva are directly correlated to those in plasma but are 70% less concentrated<sup>50</sup>. The patients were given a set of instructions and 2 saliva collection tubes (Salivette®) to collect at 12pm (baseline) and 2am.

The tubes collected from the patients were centrifuged at 3000rpm for 5 minutes. The absorptive sponge insert was discarded and 200µl was aliquoted into Eppendorf tubes. The samples and standards were then prepared using 25µl of pre-treatment solution, vortexed and incubated at RT for 10 minutes. The reaction was neutralised using 25µl of neutralising solution and the tubes were then vortexed and centrifuged for 5 minutes at 10,000rpm.

The melatonin assay is a competitive immunoassay using a capture (Ab) technique. The polyclonal Kennaway G280 anti-melatonin has been pre-coated by the manufacturer onto the microtiter plate. The plate was washed twice with 300µl of wash buffer. Immediately, 100µl of blanking reagent, pre-treated samples, zero calibrator, calibrators, low and high controls were aliquoted into each well in duplicate. The plate was incubated at 4°C for 16-24 hours. The following day, 50 µl of biotin conjugate was added to each well and the plate was incubated at room temperature on an orbital shaker for 1 minute (400-600rpm). The plate was then incubated at 4°C for 3 hours. The melatonin in the samples and controls competed with biotinylated melatonin. The plate was then washed 4 times using wash buffer, followed by 100 µl of enzyme label (streptavidin conjugated to horse radish peroxidase) added to all the wells. The enzyme was then allowed to bind to the melatonin-biotin complex at room temperature on an orbital shaker for 60 minutes (400-600rpm). The plate was then washed 4 times to remove any unbound enzyme, using wash buffer. The tetramethylbenzidine (TMB) substrate was brought to room temperature and 100 µl was added to each well. The plate was

them incubated at room temperature on an orbital shaker for 30minutes (400-600rpm). A chromophore in reverse proportion to the melatonin present was formed. The stop solution (100  $\mu$ l) was then added and the absorbance was read at 450nm.

To determine the melatonin concentration, the percentage of melatonin bound was calculated by dividing absorbance values of the known standard calibrators and multiplying the value by 100 (**Figure 29**). The percentage bound was plotted against the known concentrations of the calibrators. The sample percentage bound was also calculated by dividing the absorbance values by the zero calibrator and the melatonin concentrations were extrapolated using the standard curve.



**Figure 29.** Standard curve generated from known concentrations of melatonin vs percentage bound melatonin at different concentrations. The percentage bound was calculated by dividing the average absorbance values by the zero calibrator average absorbance values at an optical density of 450nm.

## 2.8 Statistical analysis

Statistical analysis of the results were performed using Graphpad Prism 5.0. The results are represented as mean  $\pm$  standard error of the mean (SEM). Standard curves were generated using either linear or non linear regression curves. Correlation analysis was performed using Pearson R correlation coefficients. Man-Whitney t-tests (two tailed) were performed to compare means in two different groups. The test was used because it does not assume that the data are normally distributed. Statistical significance was set at not significant (ns)  $>0.05$ , \*:p<0.05, \*\*:p<0.01 and \*\*\*:p<0.001.

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## CHAPTER 3: RESULTS

### 3.1 Characteristics of study participants

A summary of the characteristics of the study population is shown in **Table 3**. A total of 78 pregnant and postpartum women were recruited for this study (24 HC, 40 CD and 14 PPCM). Of these, there were 12 pregnant and 12 postpartum HC, 23 pregnant and 17 postpartum CD and 5 pregnant and 9 postpartum PPCM women. The pregnant PPCM group were women who were diagnosed with PPCM in a previous pregnancy and were pregnant again.

There was no statistically significant difference in the age, BP or body mass index within the groups. The mean $\pm$ SEM age in years for the HC group was 30.0 $\pm$ 1.6, the CD group 28 $\pm$ 1.4 and the PPCM group 29 $\pm$ 2.1. The mean BP in millimetres of mercury (mmHg) in the HC group was 122/74, in the CD group 120/78 and in the PPCM group 125/80. The mean $\pm$ SEM BMI in kilograms (kg) per height in centimetre (cm) squared of the HC group was 30.1 $\pm$ 3.1, the CD group 27 $\pm$ 1.3 and the PPCM group 27.8 $\pm$ 1.2. The mean $\pm$ SEM heart rate in beats per minute of the HC group was 78, the CD group was 84 and the PPCM group was 92. The PPCM group did have a significantly higher heart rate compared to the HC ( $p=0.01$ ). There was no difference between the HC and CD or CD and HC heart rate. The New York Heart Association (NYHA) functional classification (FC) system, most recently updated in 1994, is a method of classifying the effect of cardiac disease in patients in clinical practice<sup>113</sup>. The four classes and their relative objective assessments are shown in **Table 4**. The number of women in each functional class are shown in **Table 3**. The number of women who were taking ACE inhibitors, beta blockers or bromocriptine is mentioned in the table. Of the 9 postpartum PPCM women, 7 were treated with bromocriptine.

**Table 3:** Characteristic of study participants

	Total			Pregnant			Postpartum		
	HC	CD	PPCM	HC	CD	PPCM	HC	CD	PPCM
<b>N</b>	24	40	14	12	23	5	12	17	9
<b>Age (years)</b>	30.0±1.6	28.1±1.4	29.1± 2.1	31.4±2.6	27.0±2.3	28.8±3.3	29.3±2.5	29±0.7	29.1±1.1
<b>Race</b>									
African	8	14	9	4	8	3	4	6	5
Mixed	16	24	5	7	14	2	8	10	4
Caucasian	1	2	0	1	1	0	0	1	0
<b>Blood pressure (mmHg)</b>	122/74	120/78	125/80	118/71	118/75	140/81	124/76	121/71	117/75
Hypertension (n)	3	5	3	3	3	3	0	3	0
Diabetes (n)	3	4	0	2	3	0	1	1	0
Heart rate (bpm)	78.2±2.7	84±4.1	92±5.1 *	72.8±3.1	80±3.7	92	83.3±34.2	86±3.1	106±6.3 *
HIV+ (n)	3	8	3	2	2	1	1	1	2
<b>Body mass index(BMI)</b>									
Height (cm)	1.62	1.63	1.59	1.59	1.62	1.59	1.65	1.63	1.59
Weight (kg)	78.8	72.3	70.3	76.5	70.9	66.1	81.1	73.5	72.1
BMI	30.1±3.1	27.1±1.3	27.8±1.2	31.4±4.1	26.7±0.9	25.9±0.9	29.9±2.2	27.6±1.5	28.7±1.9
<b>Diagnosis</b>									
PPCM	0	0	9	0	0	0	0	0	9
Previous PPCM	0	0	5	0	0	5	0	0	0
CHD	0	20	0	0	9	0	0	11	0
RHD	0	13	0	0	8	0	0	5	0
CM	0	8	0	0	5	0	0	3	0
<b>NYHA FC</b>									
0	24	0	0	12	0	0	12	0	0
1	0	11	1	0	3	0	0	9	0
2	0	23	9	0	16	3	0	7	6
3	0	5	2	0	3	1	0	2	1
4	0	1	2	0	1	1	0	0	1
<b>Medication</b>									
ACE inhibitors	0	6	5	0	4	2	0	2	3
Beta blockers	0	5	2	0	3	0	0	2	2
Bromocriptine	0	0	7	0	0	0	0	0	7

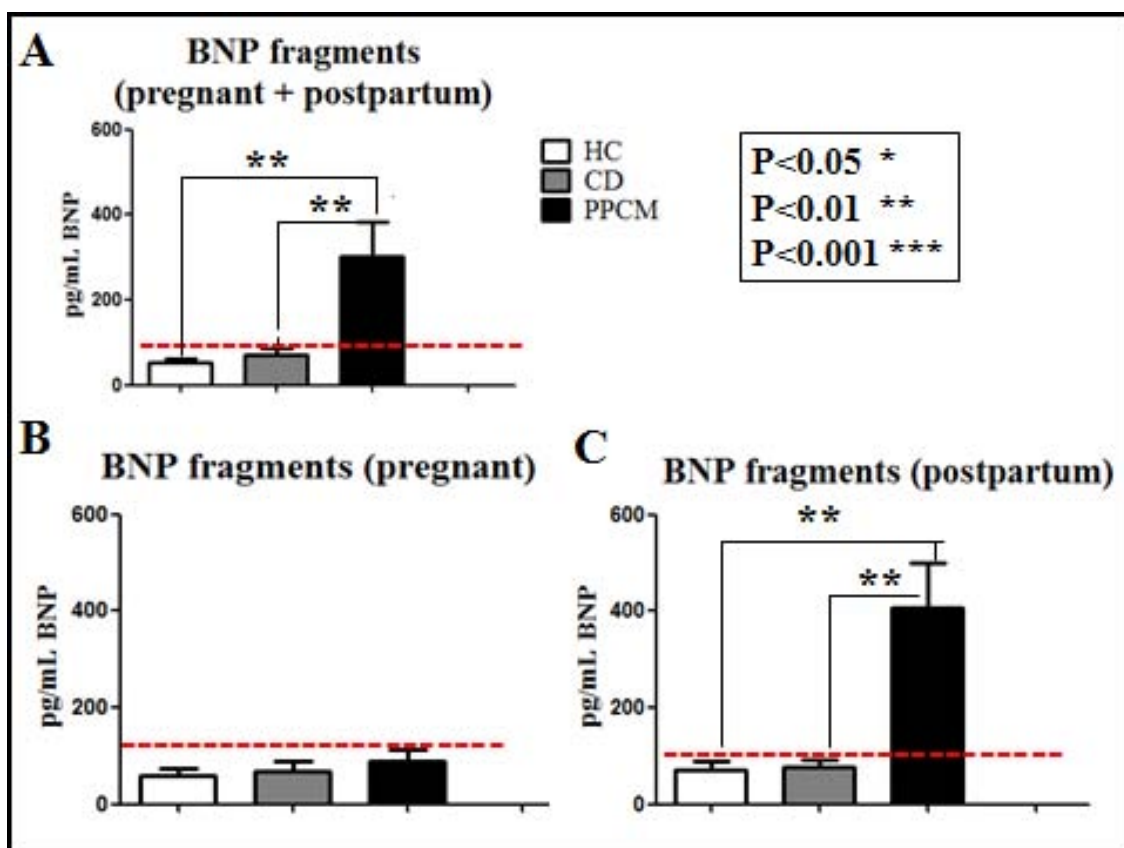
**Table 4.** The New York Heart Association (NYHA) functional classification system

The 1994 New York Heart Association (NYHA) Classification system	
Functional capacity	Objective assessment
Class I. Patients with cardiac disease but without resulting limitation of physical activity. Ordinary physical activity does not cause undue fatigue, palpitation, dyspnea, or anginal pain.	A. No objective evidence of cardiovascular disease.
Class II. Patients with cardiac disease resulting in slight limitation of physical activity. They are comfortable at rest. Ordinary physical activity results in fatigue, palpitation, dyspnea, or anginal pain.	B. Objective evidence of minimal cardiovascular disease.
Class III. Patients with cardiac disease resulting in marked limitation of physical activity. They are comfortable at rest. Less than ordinary physical activity causes fatigue, palpitation, dyspnea, or anginal pain.	C. Objective evidence of moderately severe cardiovascular disease.
Class IV. Patients with cardiac disease resulting in inability to carry on any physical activity without discomfort. Symptoms of heart failure or the anginal syndrome may be present even at rest. If any physical activity is undertaken, discomfort is increased.	D. Objective evidence of severe cardiovascular disease.

Reprinted from Nomenclature and Criteria for Diagnosis of the Heart and Great Vessels, 9th ed. Boston (MA): Little, Brown, & Co; 1994:253-6 with permission.

### 3.2 Serum concentrations of basic natriuretic peptide (BNP) fragments

The BNP fragment levels in the serum of pregnant or postpartum HC, CD and PPCM women were quantified using a BNP fragment assay. BNP levels are a useful marker of heart failure<sup>12</sup>. According to the ESC Guidelines (2012), the optimal upper cut-off/exclusion point for a healthy physiological BNP is 100pg/mL<sup>108</sup>. The results of the BNP fragment assay are shown in **Figure 30**. **Figure 30A** shows the total BNP concentration, expressed in pg/mL, in 14 pregnant and postnatal women with PPCM ( $303\pm 38.1$  pg/mL), which is significantly higher than the 34 CD ( $71.8\pm 24.4$  pg/mL,  $p=0.0019$ ) and 20 HC groups ( $52\pm 5.43$ ,  $p=0.0011$  pg/mL). The HC group was not significantly different to the CD group ( $p=0.2463$ ). There was no difference between the pregnant 10 HC ( $58.21\pm 5.15$  pg/mL), 20 CD ( $68.95\pm 25.45$  pg/mL) and 5 PPCM women ( $70.5\pm 15.36$  pg/mL) (**Figure 30B**). **Figure 30C** shows the total BNP concentration in pg/mL in postnatal women, with 9 PPCM women ( $406.05\pm 40.54$ ) presenting a significantly higher level of BNP than the CD group ( $76.61\pm 5.3$ ,  $p=0.0021$ ) and HC ( $70\pm 15.7$ ,  $p=0.0021$ ). The postpartum HC group was not significantly different to the postpartum CD group ( $p=0.9259$ ) (**figure 30C**).

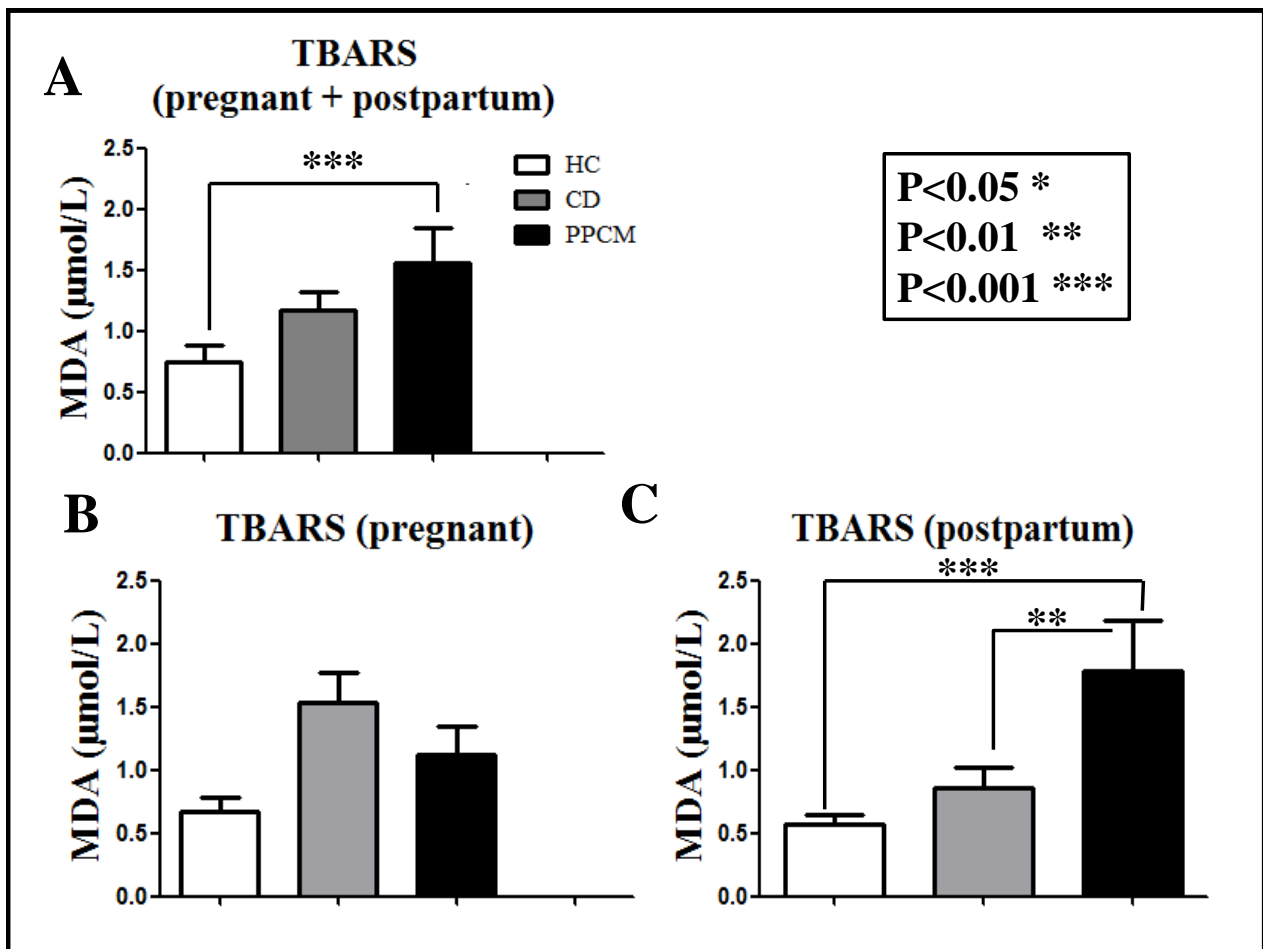


**Figure 30.** BNP fragments concentration in serum by BNP fragment ELISA. The BNP concentration in pg/mL was quantified in pregnant and postpartum healthy control (HC), with other cardiac disease (CD) and peripartum cardiomyopathy (PPCM) . Figure 30A shows the total BNP fragment concentration in the pregnant and postpartum women. Figure 30B shows the BNP fragments concentration in pregnant women. Figure 30C shows the BNP fragments concentration in postpartum women. According to ESC guidelines a BNP measurement greater than 100pg/mL is indicative of heart failure (dotted line). (ns: not significant, P<0.05 :\*,P<0.01 :\*\* and P<0.001:\*\*\*).

### 3.3 Measurement of oxidative stress levels.

To measure the oxidative stress levels in the plasma of the pregnant or postnatal HC, CD and PPCM women, a TBARS assay measuring plasma MDA concentrations was used/carried out. The results are shown in **Figure 31**. **Figure 31A** shows that the total MDA concentration expressed in  $\mu\text{mol/mL}$  in the 12 pregnant and postnatal women with PPCM ( $1.64 \pm 0.45 \mu\text{mol/mL}$ ) is significantly higher than that of the 20 HC women ( $0.75 \pm 0.3 \mu\text{mol/mL}$ ,  $p=0.0008$ ), but were not higher than that of the 34 CD women ( $1.11 \pm 0.44 \mu\text{mol/mL}$ ,  $p=0.157$ ). The MDA levels in HC women were not significantly different from the levels of the CD women ( $p=0.0821$ ). There was no difference between the MDA levels of the 10 pregnant HC ( $0.67 \pm 0.17 \mu\text{mol/mL}$ ), 23 CD ( $1.44 \pm 0.51 \mu\text{mol/mL}$ ) and 4 PPCM ( $1.16 \pm 0.24 \mu\text{mol/mL}$ ) women (**Figure 31B**). **Figure 31C** shows that the MDA concentration in the postnatal PPCM women ( $1.78 \pm 0.52 \mu\text{mol/mL}$ ) was higher than both the levels of MDA in CD ( $0.86 \pm 0.31 \mu\text{mol/mL}$ ,  $p=0.007$ ) and HC women ( $0.57 \pm 0.11 \mu\text{mol/mL}$ ,  $p=0.005$ ).

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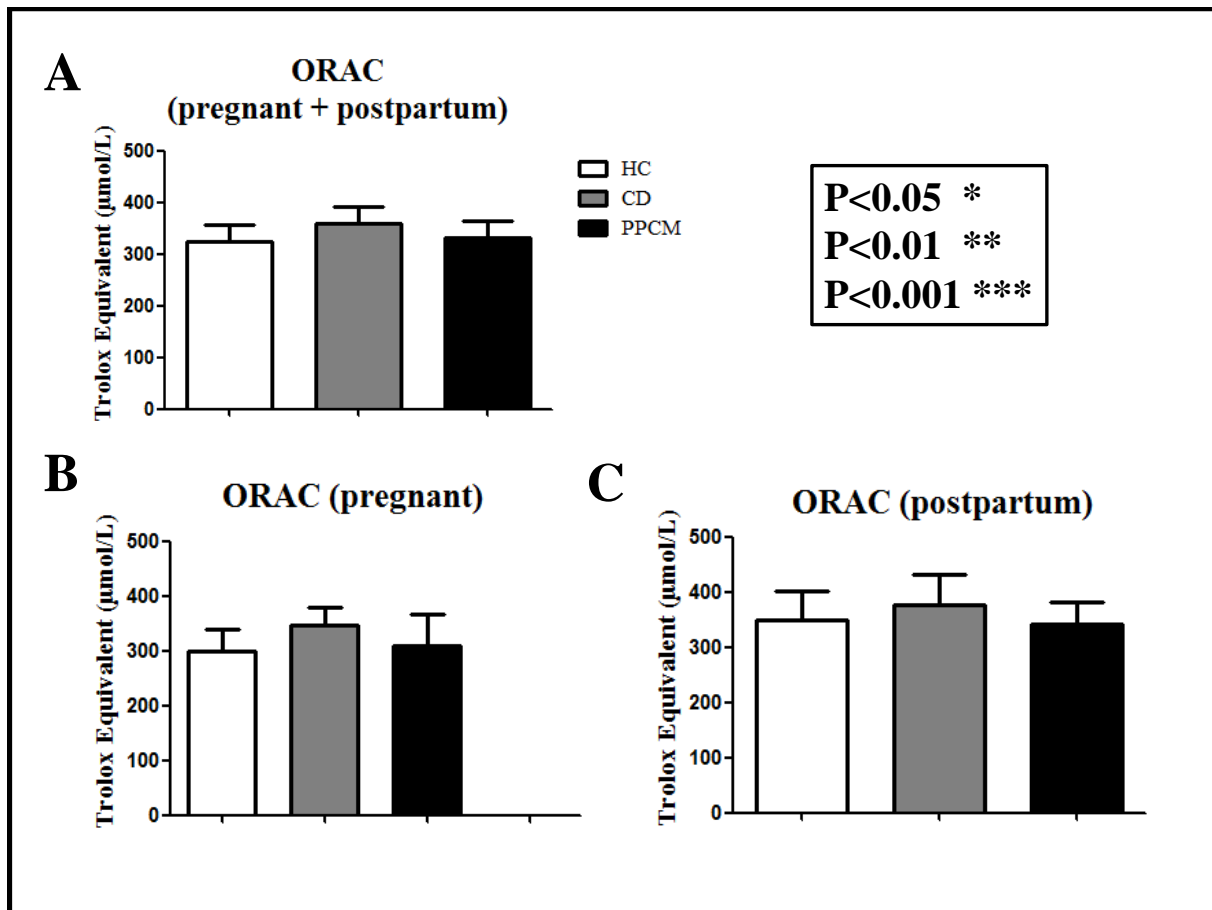


**Figure 31.** Thiobarbituric acid reactive substances assay (TBARS) assay for the measurement of malondialdehyde (MDA) in  $\mu\text{mol/L}$  plasma of healthy control (HC), with other cardiac disease (CD) and peripartum cardiomyopathy (PPCM). Figure 31A: The MDA concentration in both pregnant and postpartum women. Figure 31B: The MDA concentration in the pregnant women only. Figure 31C: The MDA concentration in the postpartum women only. (ns: not significant,  $P < 0.05$  :\*,  $P < 0.01$  :\*\*, and  $P < 0.001$  :\*\*\*)

### 3.4 The analysis of plasma antioxidant capacity

The total antioxidant capacity of the plasma from the HC, CD and PPCM pregnant and postpartum women was assessed by using the ORAC assay (**Figure 32**). The 20 pregnant and postpartum PPCM women had a total antioxidant capacity of  $332.3 \pm 47.3$   $\mu\text{mol/L}$  trolox equivalents (TE). There was no difference between the pregnant and postnatal PPCM women compared to the CD women ( $359.9 \pm 65.5$   $\mu\text{mol/L}$ ,  $p=0.6187$ ) or the HC women ( $321.9 \pm 64.5$   $\mu\text{mol/L}$ ,  $p=0.6926$ ) (**Figure 32A**). There was also no difference between the CD and HC women ( $p=0.3286$ ). The ORAC assay did not differ between the pregnant PPCM women ( $365.4 \pm 44.3$   $\mu\text{mol/L}$ ) and the pregnant CD ( $343.2 \pm 54.1$   $\mu\text{mol/L}$ ,  $p=0.666$ ) or the pregnant HC women ( $312.9 \pm 55.2$   $\mu\text{mol/L}$ ,  $p=0.6$ ) (**Figure 32B**). There was also no difference between the CD and HC women ( $p=0.2105$ ). A similar trend was observed in the postnatal PPCM women ( $347.7 \pm 52.3$   $\mu\text{mol/L}$ , compared to the postnatal CD women ( $376.7 \pm 79$   $\mu\text{mol/L}$ ,  $p=0.8665$ ) or the postnatal HC women ( $347.7 \pm 52.3$   $\mu\text{mol/L}$ ,  $p=1$ ) (**Figure 32C**).

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**Figure 32.** Oxygen radical absorbance capacity(ORAC) assay to determine the plasma antioxidant capacity in Trolox equivalents( $\mu\text{mol/L}$ ) in pregnant and postpartum healthy control (HC), with other cardiac disease (CD) and peripartum cardiomyopathy PPCM) women. Figure 32A shows both pregnant and postpartum antioxidant capacity. Figure 32B shows the antioxidant capacity in pregnant women. Figure 32C shows the antioxidant capacity in postpartum women. (ns: not significant,  $P<0.05$  :\*, $P<0.01$  :\*\* and  $P<0.001$ :\*\*\*).

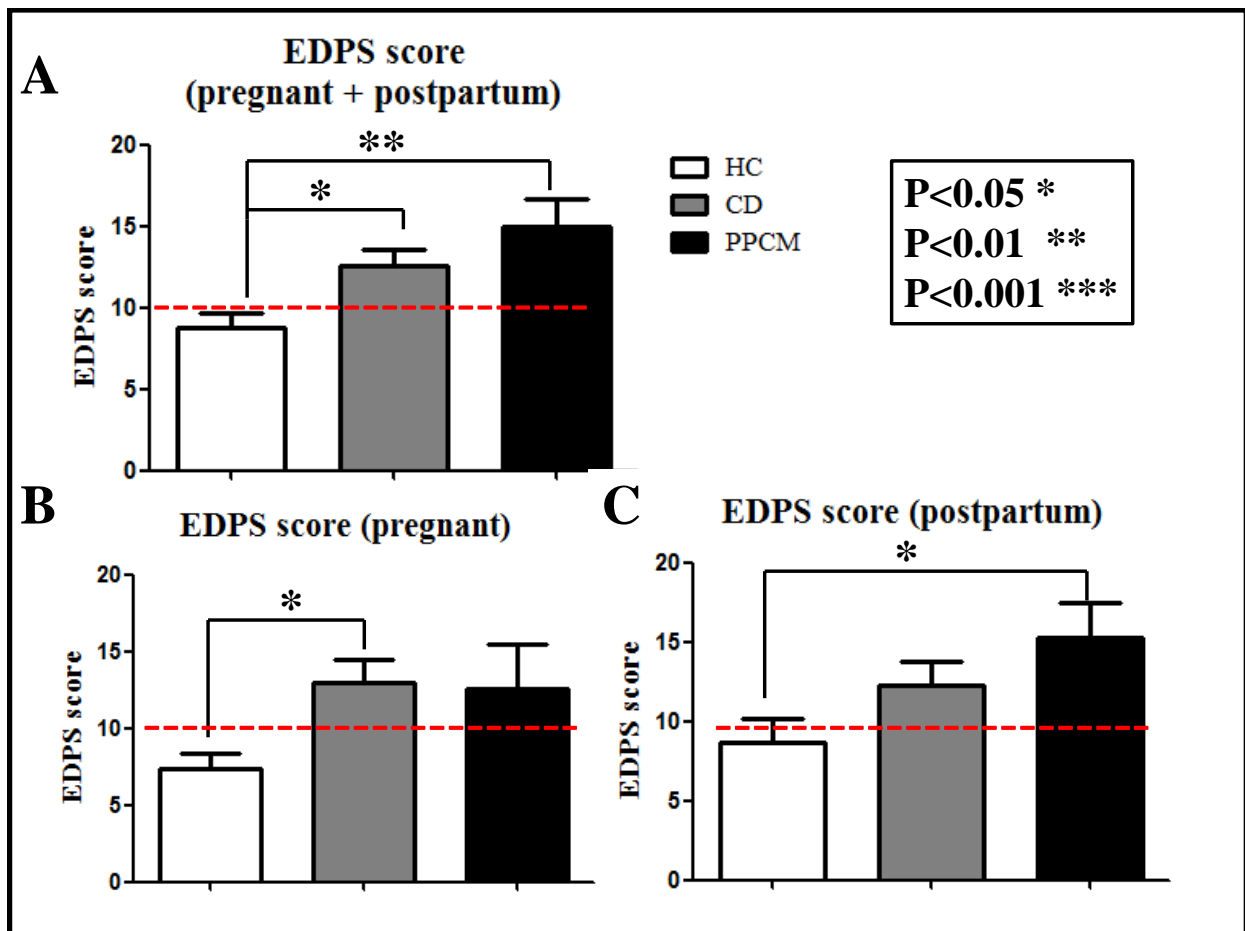
### 3.5 Edinburgh postnatal depression scale (EDPS)

To screen for possible depression, the EPDS was administered to the pregnant and postpartum HC, CD and PPCM women. **Figure 33A** displays the EDPS score of both the pregnant and postnatal women. The mean EDPS score of the HC women was  $8.79 \pm 2.5$ , the mean score of the CD women was  $12.56 \pm 3.3$  and the mean score of the PPCM women was  $14.69 \pm 3.3$ . There was a significant difference ( $p=0.0078$ ) between the HC and PPCM group, as well as between the HC and CD group ( $p=0.0356$ ). There was no significant difference between the CD and PPCM group ( $p=0.351$ ).

The pregnant HC women had a mean score of  $8.08 \pm 2.3$ , the pregnant CD women had a mean score of  $13.07 \pm 3.2$  and the pregnant PPCM women had a mean score of  $13.5 \pm 3.5$  (**Figure 33B**). The pregnant CD group had a significantly higher score than the HC group ( $p=0.0443$ ). There was no significant difference between the pregnant PPCM and the CD group ( $p=0.1959$ ) or the HC group ( $p=0.8862$ ).

The postpartum HC women had a mean score of  $8.7 \pm 2.5$ , the CD women had a mean score of  $12.23 \pm 3.4$  and the PPCM women had a mean score of  $15.22 \pm 3.3$  (**Figure 33C**). The postpartum PPCM women had a significantly higher score than the postpartum HC women ( $p=0.0297$ ). The CD women did not have a different score compared to the PPCM women ( $p=0.2137$ ) or the HC women ( $p=0.1755$ ).

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**Figure 33.** Edinburgh postnatal depression scores (EDPS) in HC, CD and PPCM women. Figure 33A. EDPS of all HC, CD and PPCM women. Figure 33B EDPS scores of pregnant HC,CD and PPCM women. Figure 33C EDPS scores of postpartum HC, CD and PPCM women. The recommended cut-off for possible depression is a score of 10 or more(dotted line) ( ns: not significant, P<0.05 :\*,P<0.01 :\*\*, and P<0.001:\*\*\*)

### 3.6 Sleep quality questionnaire results

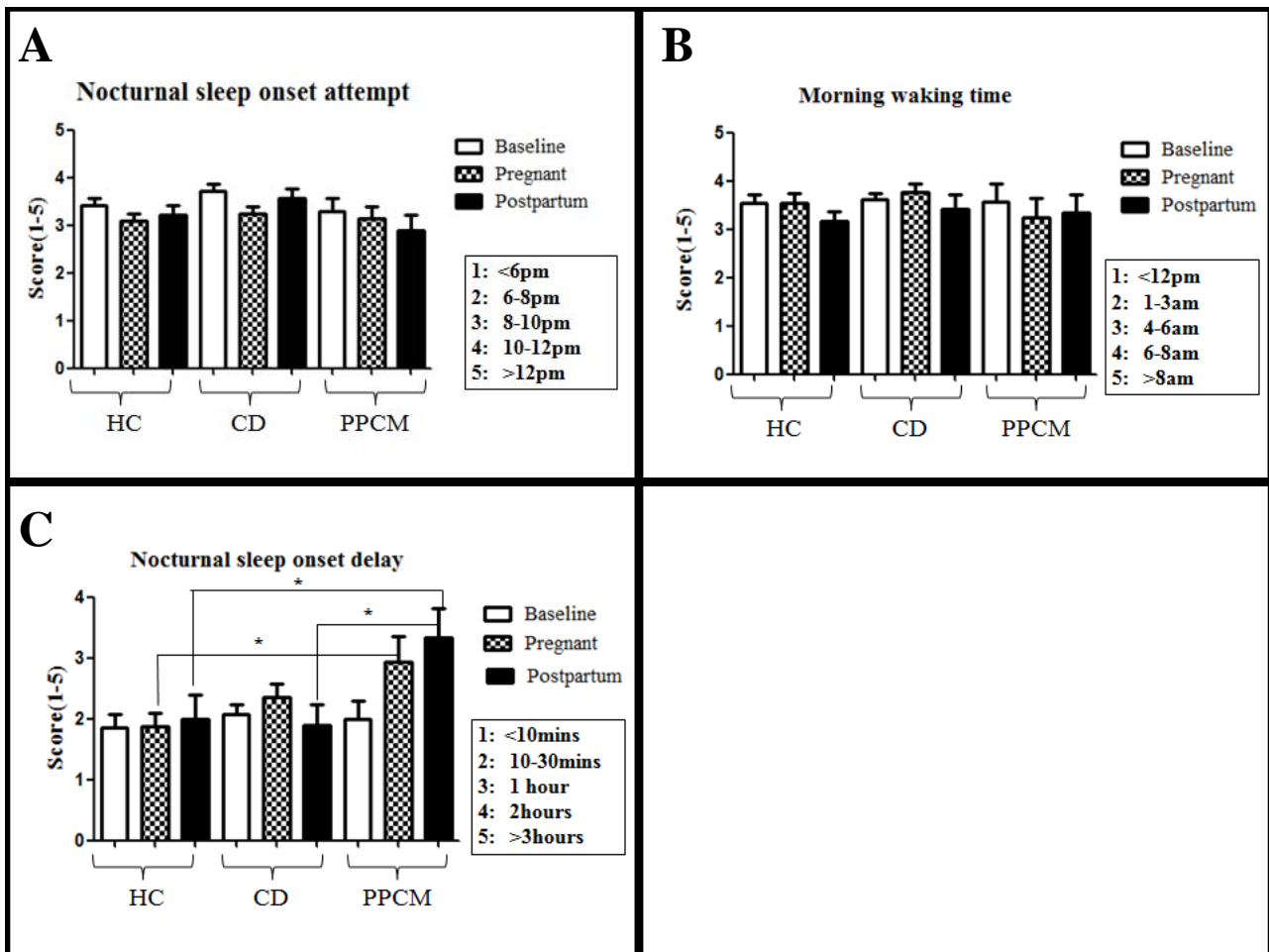
#### Question 1-3: Time of onset of sleep and waking

The results of the mean onset of sleep are shown in **Figure 34A**. There was no significant difference between the sleep onset time of the HC, CD or PPCM women before, during or after pregnancy.

The results of the mean morning waking time are shown in **Figure 34B**. There was no significant difference between the sleep onset time of the HC, CD or PPCM women at any time point.

The results of the mean sleep onset time are shown in **Figure 34C**. There was no significant difference in the baseline sleep onset time between the groups. The PPCM women had a significantly longer sleep onset time compared to the HC during pregnancy and postpartum ( $P < 0.05$ ). They also had a significantly long sleep onset time compared to the CD group postpartum. There was, however, no difference between the PPCM sleep onset time compared to the CD group during pregnancy.

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**Figure 34.** Figure 34A: The mean score(1-5) of sleep onset time, 1(early)-5(late) before during and after pregnancy in the HC, CD and PPCM women. Figure 34B: The mean score(1-5) of morning, 1(early)-5(late) before during and after pregnancy in the HC, CD and PPCM women. Figure 34C: The mean score(1-5) of nocturnal sleep onset delay, 1(fast)-5(delayed) before during and after pregnancy in the HC, CD and PPCM women. (ns: not significant,  $P < 0.05$  :\*,  $P < 0.01$  :\*\*, and  $P < 0.001$  :\*\*\*)

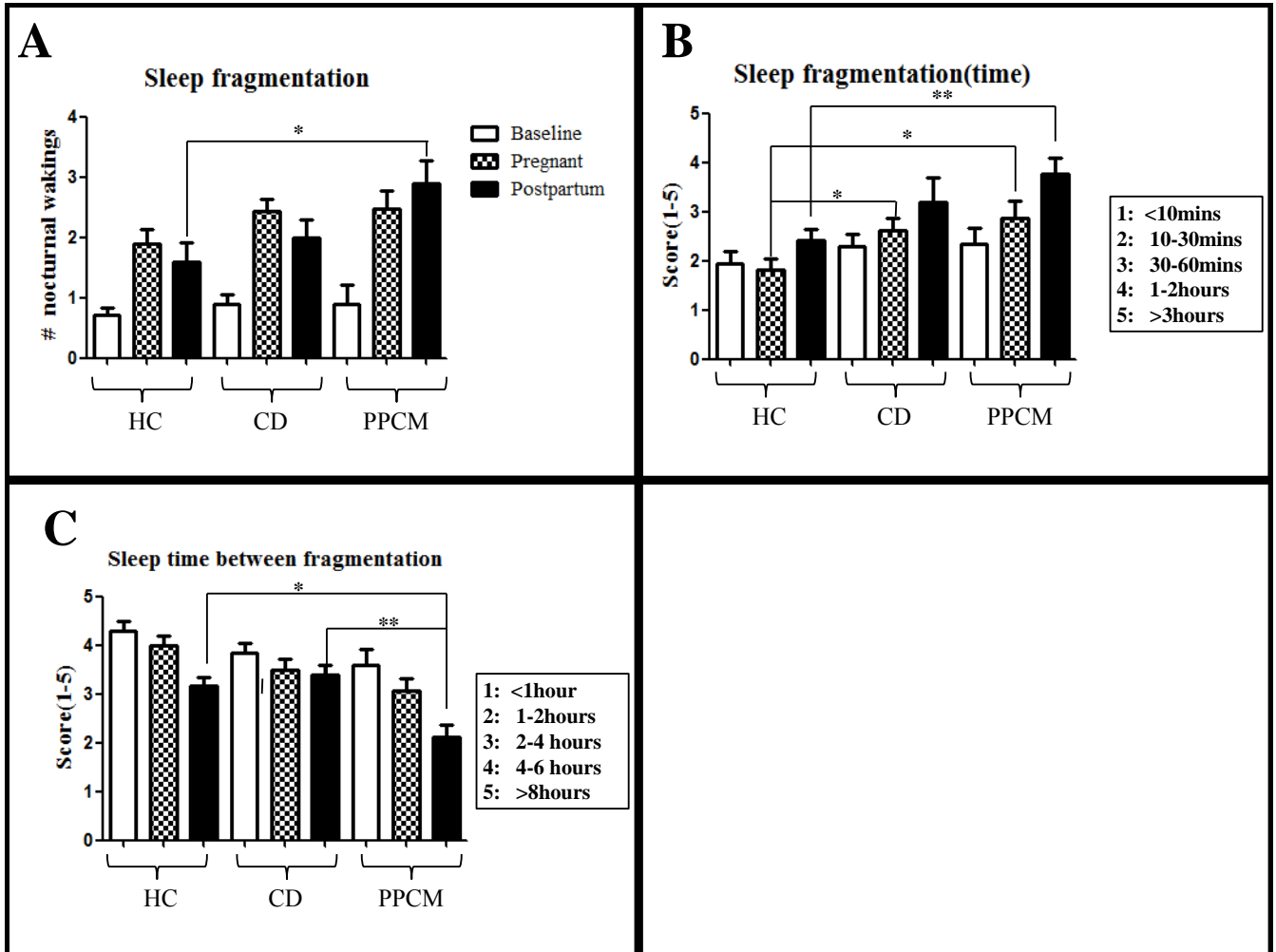
#### Question 4-6: Sleep disruption

**Figure 35A** shows the number of times the women woke up at night (sleep fragmentation). The reported sleep fragmentation of the postpartum PPCM women was significantly higher ( $P < 0.05$ ), compared to the HC group. There was a significant increase of sleep fragmentation postpartum and during pregnancy within each group, compared to the 6 months before pregnancy (baseline).

The time spent awake between awakening (sleep fragmentation time) is represented in **Figure 35B**. The pregnant ( $P < 0.05$ ) and postpartum ( $P < 0.01$ ) PPCM women had a significantly longer time of wakefulness between waking up during the night, compared to HC women. There was also a significant increase of waking time during pregnancy in the CD, compared to the HC women ( $P < 0.05$ ).

The total sleep time between the fragmentation is represented in **Figure 35C**. The postpartum PPCM women had a significantly lower sleeping time between fragmentation compared to the CD ( $P < 0.01$ ) and HC ( $P < 0.05$ ).

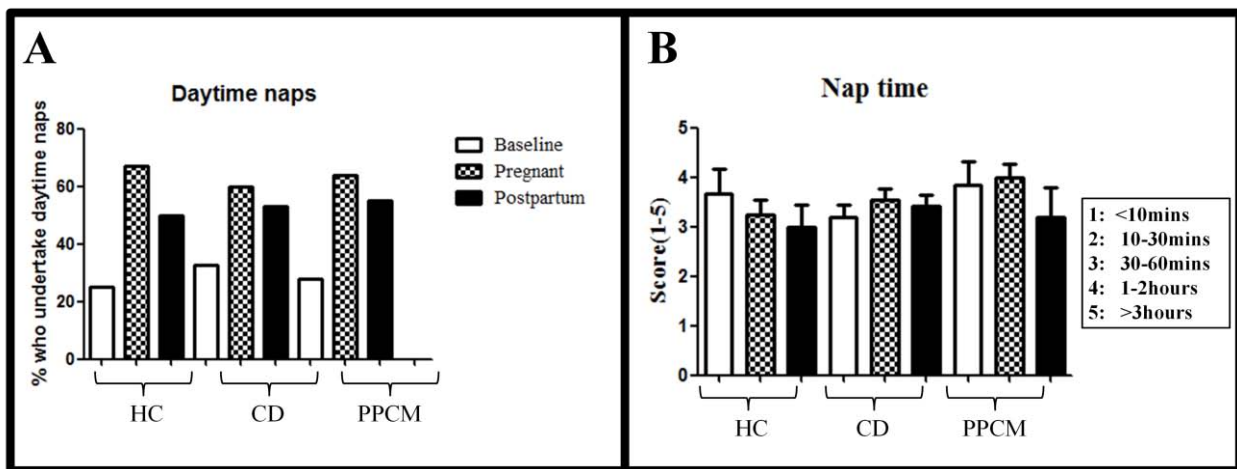
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**Figure 35.**Figure 35A: The mean number of nocturnal waking before during and after pregnancy in the HC, CD and PPCM women. Figure 35B: The mean score (1-5) of morning, 1(short)-5(long) time of being awake during night between fragmentation before during and after pregnancy in the HC, CD and PPCM women. Figure 35C: The mean score(1-5) of 1(short)-5(long) of the sleep time between fragmentation before during and after pregnancy in the HC, CD and PPCM women. (ns: not significant,  $P < 0.05$  :\*,  $P < 0.01$  :\*\*, and  $P < 0.001$  :\*\*\*)

### Question 7-8: Daytime napping

**Figure 36A** represents the percentage of HC, CD and PPCM women who undertake naps during the day. The percentage of women who took daytime naps increased significantly in each group in the final month of pregnancy and postpartum. However, there was no significant difference amongst the groups with regards to the percentage of women who took daytime naps. The total amount of daytime sleeping or napping is represented in **Figure 36B**. There was also no difference in total nap time between the groups of those women who took daytime naps.

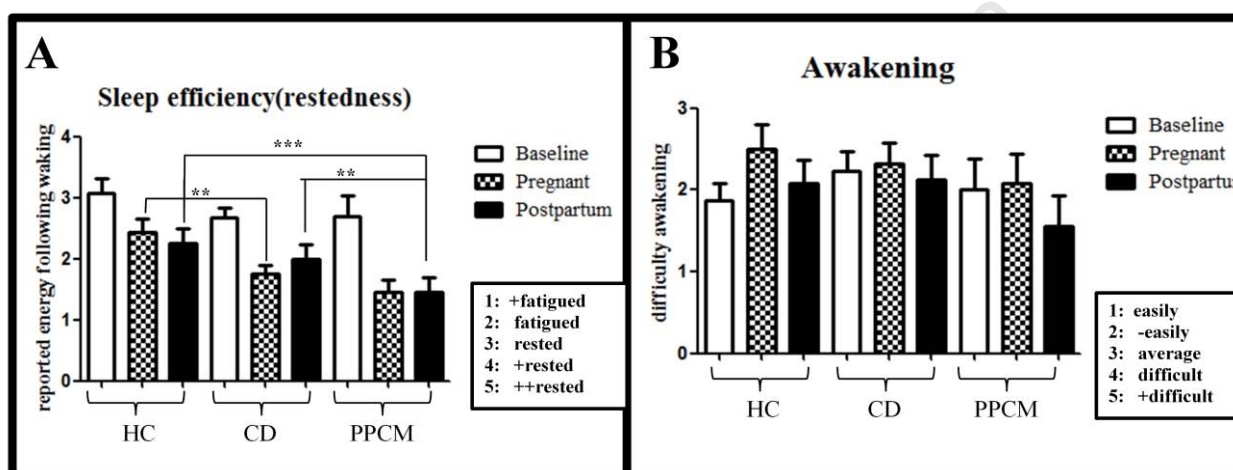


**Figure 36** Figure 36A: The percentage of women who took daytime naps before during and after pregnancy in the HC, CD and PPCM women. Figure 36B: The mean score(1-5) 1(short)-5(long) of total time of daytime naps before during and after pregnancy in the HC, CD and PPCM women. (ns: not significant,  $P < 0.05$  :\*,  $P < 0.01$  :\*\*, and  $P < 0.001$  :\*\*\*)

### Question 9-10: Sleep efficiency and ease to awake

**Figure 37A** represents the reported energy following waking. There was a significant general decrease in sleep efficiency across all groups (HC, CD and PPCM) during pregnancy and postpartum compared to baseline. The postpartum PPCM women reported a significant decrease in sleep efficiency compared to CD ( $P<0.01$ ) and HC women ( $P<0.001$ ). The CD pregnant women also reported a significant decrease in sleep efficiency during pregnancy compared to HC women ( $P<0.01$ ).

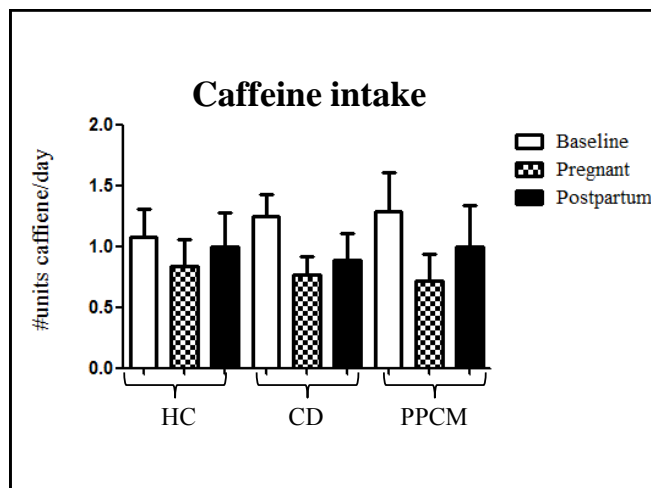
**Figure 37B** represents the ease to awake. There was no difference observed between the groups.



**Figure 37.** Figure 36A Reported energy following waking before pregnancy (baseline), during pregnancy and postpartum in the HC, CD and PPCM women (1: very fatigued, 2: fatigued, 3: rested, 4: well rested, 5: very well rested). **Figure 36B** Reported difficulty waking before pregnancy (baseline), during pregnancy and postpartum in the HC, CD and PPCM women (1: easy, 2: less easy, 3: average, 4: difficult, 5: very difficult). (ns: not significant,  $P<0.05$  :\*,  $P<0.01$  :\*\* and  $P<0.001$  :\*\*\*)

### Question 11 : Caffeine intake

There was no difference in the reported caffeine consumption during pregnancy or postpartum between the HC, CD and PPCM women (**Figure 38**). There was a minor (but not significant) decrease in caffeine consumption across all groups during pregnancy compared to baseline.

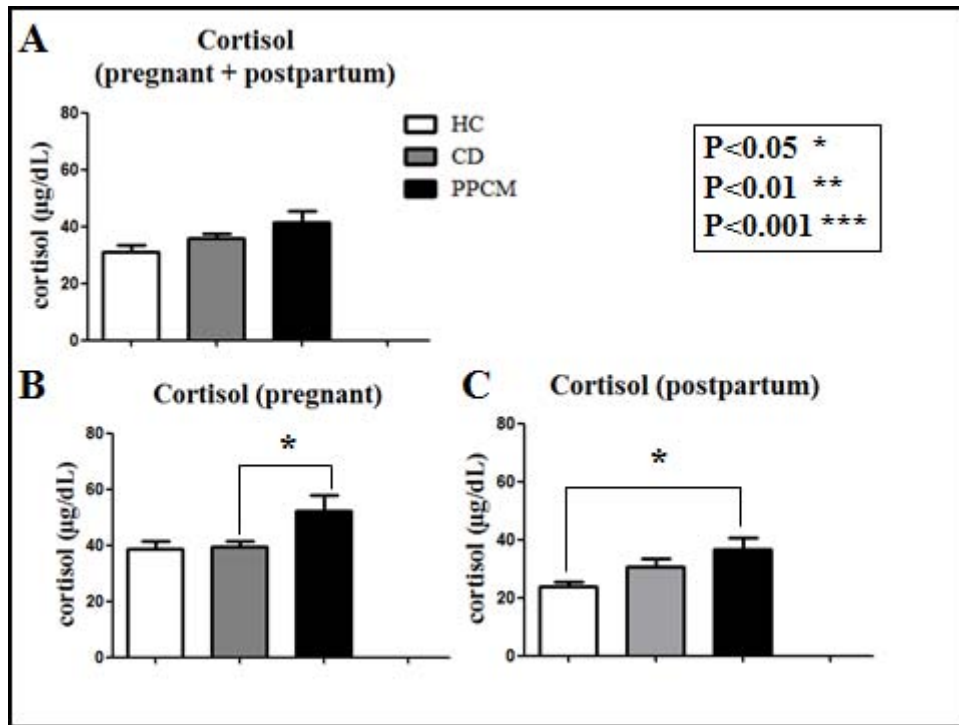


**Figure 38.** Reported mean units of caffeine (coke/coffee) per day before pregnancy(baseline), during pregnancy and postpartum in the HC, CD and PPCM women. ( ns: not significant,  $P < 0.05$  :\*,  $P < 0.01$  :\*\*, and  $P < 0.001$  :\*\*\*)

### 3.7 Cortisol quantification by Enzyme-linked immunosorbent assay (ELISA)

The serum cortisol levels in the HC, CD and PPCM women were quantified using an ELISA assay. The mean cortisol levels of the pregnant and postpartum women are shown in **Figure 39A**. The mean cortisol levels of the pregnant women are shown in **Figure 39B** and the mean cortisol levels of postpartum women are shown in **Figure 39C**. The mean cortisol concentration in the HC pregnant women was  $38.5 \pm 4.62$   $\mu\text{g/dL}$ . The mean cortisol concentration in the HC postpartum women was  $23.7 \pm 2.5$   $\mu\text{g/dL}$ . Previous studies have reported a 2-3 fold increase in serum cortisol in the final trimester, which returned to normal 3 days following childbirth ( $16\text{-}25$   $\mu\text{g/dL}$ )<sup>91</sup>. The pregnant CD women had a mean cortisol level of  $39.3 \pm 4.53$   $\mu\text{g/dL}$ . The pregnant CD women did not have significantly different cortisol concentrations compared to the HC pregnant women ( $p=0.708$ ). The pregnant PPCM women had mean cortisol concentration of  $52.46 \pm 5.4$   $\mu\text{g/dL}$ . The pregnant PPCM women did not have significantly elevated cortisol compared to HC women ( $p=0.054$ ) but were significantly higher than the CD women ( $p=0.025$ ).

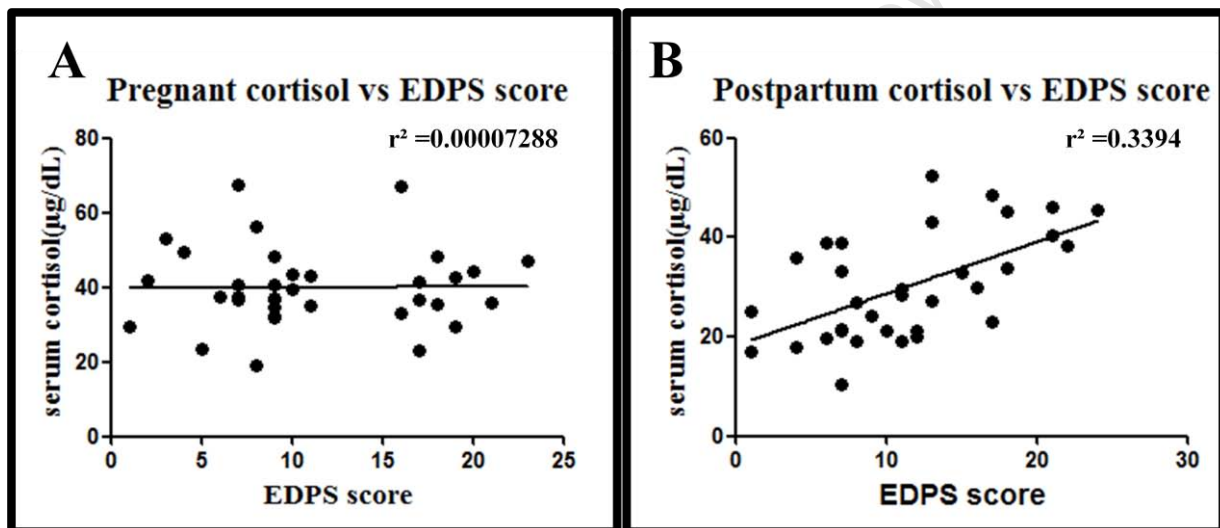
The postpartum CD women had a mean cortisol concentration of  $30.58 \pm 5.47$   $\mu\text{g/dL}$ , which was not significantly different from the HC women ( $p=0.095$ ). The postpartum PPCM women had a mean cortisol concentration of  $36.72 \pm 5.54$   $\mu\text{g/dL}$ . The postpartum PPCM cortisol was not significantly elevated compared to the CD women ( $p=0.219$ ), but was significantly elevated compared to the postpartum HC women ( $p=0.022$ ).



**Figure 39.** Cortisol quantification in diluted human serum by ELISA. The cortisol concentration in µg/dL was quantified in pregnant and postpartum healthy control(HC), with other cardiac disease(CD) and peripartum cardiomyopathy(PPCM) women. Figure 39B shows the cortisol concentration in the pregnant women. Figure 39C shows the concentration in postpartum women. (ns: not significant, P<0.05 :\*,P<0.01 \*\*: and P<0.001:\*\*\*)

### 3.8 Correlation analysis of EDPS scores and cortisol

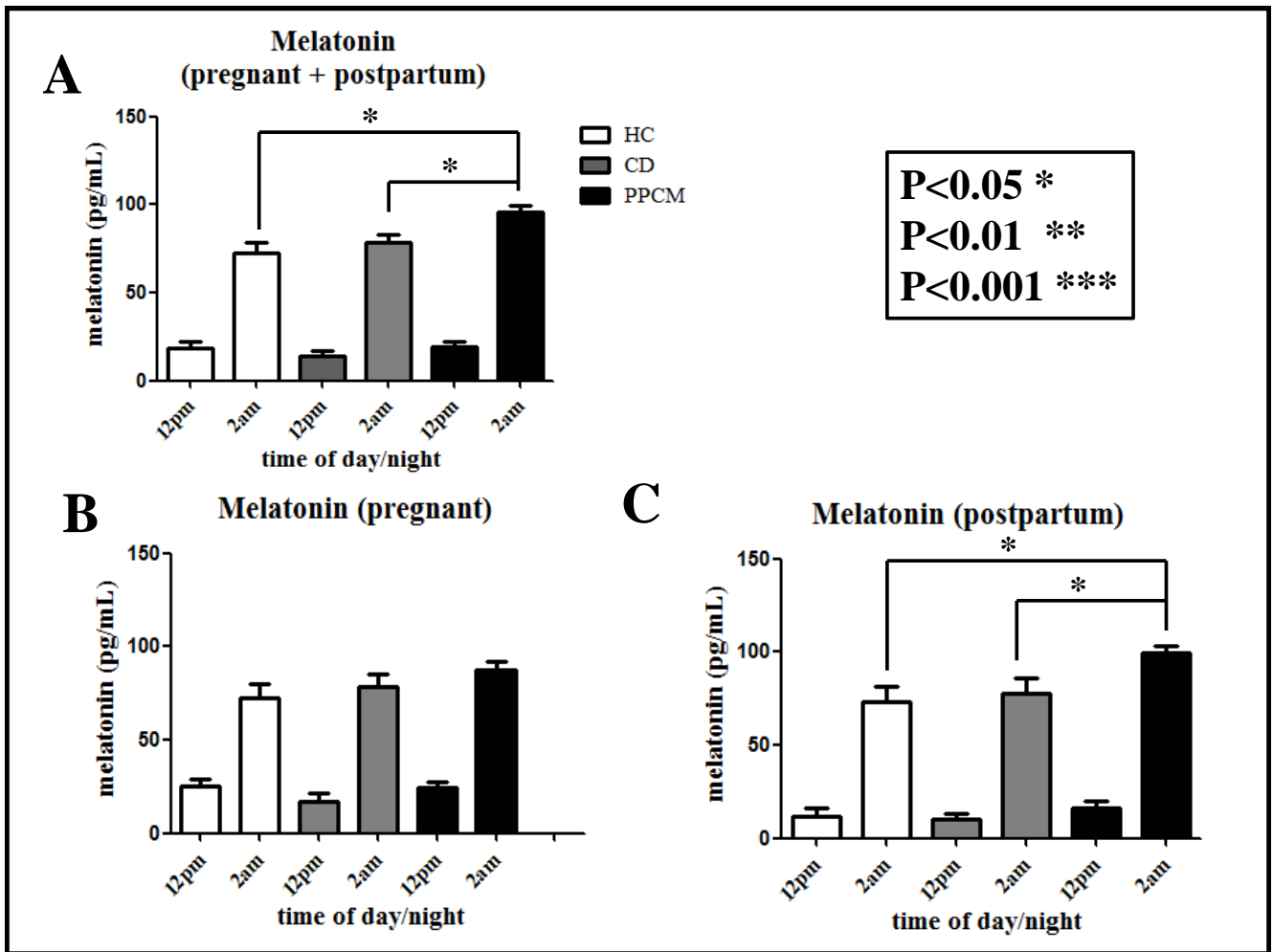
A correlation analysis was performed to compare HC, CD and PPCM EDPS scores with their individual serum cortisol concentrations. **Figure 40A** shows the correlation analysis of the serum cortisol (y- axis) vs EDPS score (x-axis) in the HC, CD and PPCM pregnant group. No correlation was found between the depression score and the cortisol level in the pregnant women. **Figure 40B** shows the correlation analysis of the serum cortisol (y-axis) vs EDPS score (x-axis) in the HC,CD and PPCM postpartum women. There was a mild positive correlation ( $r^2=0.3394$ ) between the scores and the cortisol levels in the postpartum women.



**Figure 40.** Figure 40A Correlation analysis between the pregnant group serum cortisol(y-axis) and EDPS score(x-axis). Figure 40B Correlation analysis between the postpartum group serum cortisol(y-axis) and EDPS score(x-axis).

### 3.9 Saliva melatonin quantification (ELISA)

The nocturnal (2am) and daytime (baseline) salivary melatonin concentration was determined in the pregnant and postnatal HC, CD and PPCM women by ELISA. There was a significant difference in the melatonin levels during the daytime compared to nocturnal ( $p < 0.0001$ ) in each group of women (**Figure 41A**). The combined pregnant and postnatal PPCM women had significantly higher nocturnal melatonin ( $95.7 \pm 3.4$  pg/mL) compared to the CD ( $78.3 \pm 4.9$  pg/mL) ( $p = 0.01$ ) and HC women ( $72.8 \pm 5.4$  pg/mL) ( $p = 0.0161$ ) (**Figure 41A**). The daytime melatonin levels of the pregnant and postpartum PPCM women ( $19.2 \pm 2.9$  pg/mL) were also slightly, but not significantly, elevated compared to the pregnant and postpartum CD ( $13.8 \pm 2.9$  pg/mL) or HC women ( $18.5 \pm 2.9$  pg/mL) (**Figure 41A**). There was no observable difference between the pregnant HC ( $72.9 \pm 7.9$  pg/mL), CD ( $78.7 \pm 2.9$  pg/mL) and PPCM ( $87.5 \pm 4.6$  pg/mL) women's nocturnal melatonin concentrations (**Figure 41B**). There was also no observable difference in daytime melatonin level between all the groups. The postpartum PPCM women ( $99 \pm 3.6$  pg/mL) had significantly higher nocturnal melatonin compared to the CD ( $77.8 \pm 5.2$  pg/mL) ( $p = 0.0426$ ) and HC ( $73.6 \pm 4.5$  pg/mL) ( $p = 0.0381$ ) women. The daytime melatonin levels were also slightly elevated, although not significantly (**Figure 41C**).



**Figure 41.** Figure 41A. Daytime and nocturnal salivary melatonin concentrations in HC,CD and PPCM pregnant and postpartum. . Figure 41B. Daytime and nocturnal salivary melatonin concentrations in HC,CD and PPCM pregnant. . Figure 41C. Daytime and nocturnal salivary melatonin concentrations in HC,CD and PPCM postpartum. ( ns: not significant, P<0.05 :\*,P<0.01 :\*\* and P<0.001:\*\*\*)

## CHAPTER 4: DISCUSSION

### 4.1 Summary of results

The aim of this study was to determine a possible disruption in the antioxidant melatonin levels in women with PPCM. The study also investigated whether disturbed sleeping patterns and depression may possibly contribute to the development of PPCM. A brief summary of the results are shown in **Figure 42**. The results demonstrate an increase in the marker of heart failure, BNP, in postnatal PPCM women compared to both CD and HC women. The PPCM women also demonstrated higher postpartum nocturnal melatonin levels compared to the HC and CD groups. The oxidative stress, in the form of lipid peroxidation, was also elevated in the postnatal PPCM compared to HC and CD women. Conversely, there was no observable difference in the antioxidant capacity, in the form of trolox equivalents, between the groups. The PPCM and CD groups both demonstrated increased EDPS scores and elevated serum cortisol in comparison to the HC women. The PPCM group did not demonstrate a significant difference in their sleep onset attempt (bedtime) and morning waking. However, they reported a significantly longer sleep onset delay. The postnatal PPCM group also reported higher sleep fragmentation, as well as shorter periods between waking. There was no difference in ease to wake up between the groups, but the PPCM women did report significantly lower sleep efficiency (they felt more fatigued following waking).

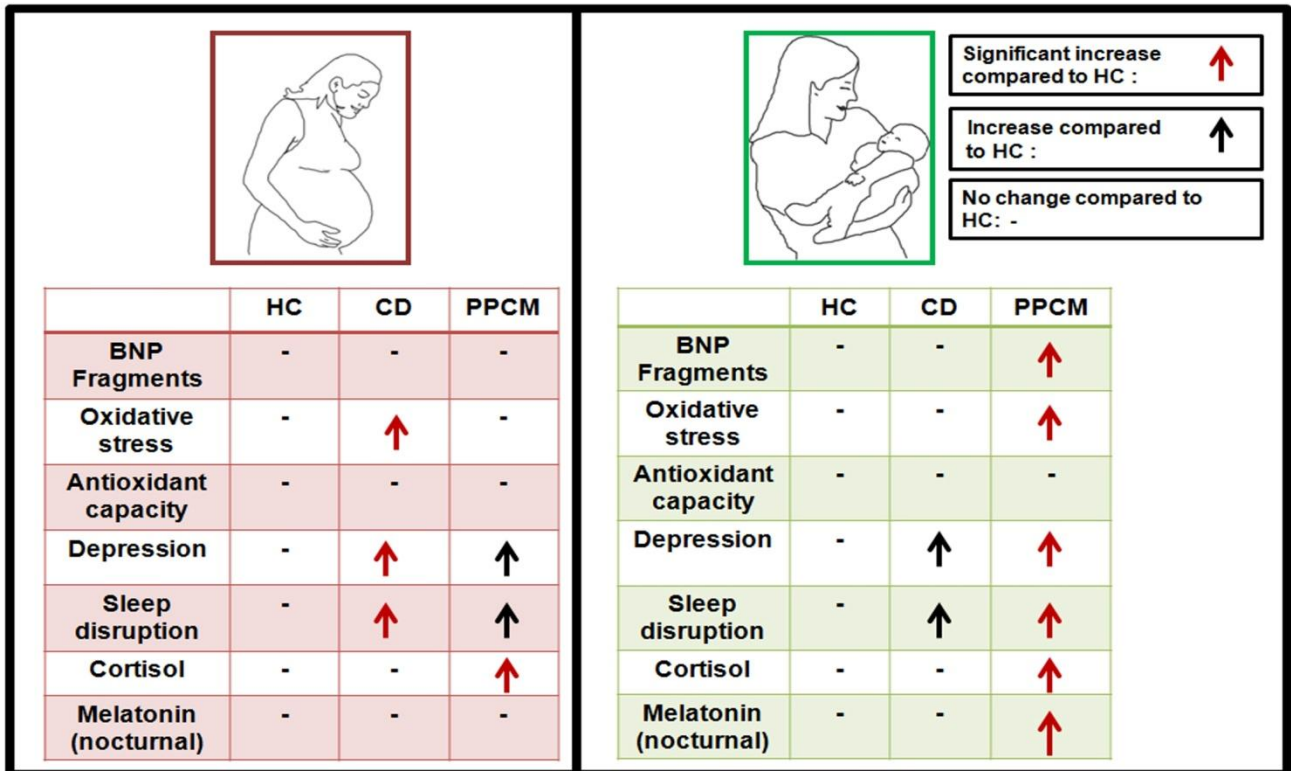


Figure 42. Summary of results in the pregnant women (left) and postnatal women (right).

#### **4.2 The serum BNP concentration is elevated in the postpartum women with PPCM**

Elevated levels of serum BNP or NT-pro BNP are established markers of heart failure<sup>12</sup>. BNP has an important role in the regulation of cardiovascular and renal homeostasis, as well as in the regulation of fatty acid metabolism and body weight<sup>12</sup>. The serum concentrations of BNP rise in pathological states, such as in ventricular dilation or reduced clearance of peptides (renal failure)<sup>12</sup>. Studies have shown a direct correlation between cardiac damage and BNP concentration<sup>12</sup>. A study by Sliwa et al. demonstrated an increase in the NT-pro BNP at baseline in postpartum women with PPCM<sup>17</sup>.

In the present study, the BNP levels in the serum of pregnant and postpartum HC, CD and PPCM women were quantified. The ESC guideline cut-off for healthy non-cardiac failure BNP levels are 100pg/mL<sup>108</sup>. The results of our study showed an increase in the BNP levels in the postpartum women with PPCM. The mean BNP concentration in the PPCM group was above the guideline cut off for normal BNP concentrations. There was a large variation in the PPCM group, most likely due to a large variation in the severity of HF. There was also a large variation in BNP levels of CD women, due to the large spectrum of their conditions. The levels of BNP in the CD women, may be lower due to their careful monitoring and treatment during pregnancy. There was no difference in the BNP levels of the pregnant PPCM women compared to the CD or HC women. This may be due to the fact that the women were previously diagnosed with PPCM and monitored and treated carefully during the subsequent pregnancy.

These results reinforce the previous studies and indicate that postpartum women diagnosed with PPCM have an increase in BNP levels as a result of heart failure due to LV dilation. Some women with PPCM do recover normal LV function and their hearts return to normal<sup>26</sup>. The discrepancy in severity also possibly explains why some women with PPCM recover LV function and their hearts return to normal and some women do not recover and are at risk for reoccurrence.

#### **4.3 The serum MDA, a product of lipid peroxidation is elevated in the postpartum PPCM group.**

Oxidative stress has been shown to play a central role in the pathogenesis of cancer, atherosclerosis, aging and other chronic diseases<sup>40</sup>. Sustained increases in oxygen radical production in the mitochondria may lead to a destructive cycle of mitochondrial DNA damage, as well as functional decline, further ROS generation and cellular injury<sup>42</sup>. A study conducted by Hilfiker-Kleiner et al. found that, in a mouse model of PPCM with a cardiomyocyte specific deletion in STAT3, they had increased oxidative stress and cathpepsin D<sup>18</sup>. This oxidative stress mediates the cathpepsin D cleavage of the nursing hormone, prolactin, into a 16kDa pro apoptotic form and develop the hallmarks of PPCM<sup>18</sup>. Hilfiker-Kleiner et al. performed further studies in humans and demonstrated a reduction in the STAT-3 protein in the heart tissue of women with PPCM, in comparison to healthy controls<sup>19</sup>. In the present study, the oxidative status in the plasma of the postpartum and pregnant women with PPCM was compared to that of the CD and HC controls. The ROS and RNS demonstrated very short half lives. It is therefore preferable to measure a decrease in total antioxidant capacity or by the estimation, the products of oxidation of lipids or DNA such as MDA and isoprostanes<sup>33 109</sup>. The present study utilised the TBARS assay to measure the lipid peroxidation product, MDA, as well as to assess the antioxidant capacity compared to the vitamin E analogue, trolox, using the ORAC assay.

The MDA concentration in the pregnant and postnatal women combined was higher in the PPCM women than in the HC, but not the CD women. The average value for healthy non-pregnant subjects reported by Jentzsch et al. was  $0.47 \pm 0.12 \mu\text{mol/l}$ <sup>110</sup>. Our HC values were close to this range but the PPCM group values were considerably higher. The pregnant PPCM group was not significantly different compared to the pregnant HC and CD group. The CD group did appear elevated compared to the HC and PPCM during pregnancy, but not significantly. This may be due to other factors such as elevated lipids or other causes of oxidative stress in the various CD women. In contrast, MDA concentration was significantly higher in the postpartum PPCM women compared to both the CD and HC. This suggests that there is an increase in oxidative stress in the form of lipid peroxidation specific to the postpartum PPCM women. This reflects the observation by Hilfiker-Kleiner et al., when the women with PPCM studied demonstrated an increase in oxLDL compared to controls<sup>19</sup>.

Oxidative stress increases in the final trimester of pregnancy, however, this returns to normal in healthy postpartum women<sup>19</sup>. The oxidative process during pregnancy has a regulatory function and

involves the ROS and RNS metabolic pathway activation during early pregnancy to facilitate pregnancy related changes<sup>28</sup>. The increase in oxidative stress in healthy women peaks during the second trimester<sup>38</sup>. This early physiological increase in oxidative stress may be the reason why previous studies such as the VIP trial, using antioxidants in high concentrations to prevent pre-eclampsia, failed<sup>43</sup>. These high concentrations may even worsen increased oxidative stress later in pregnancy as the high doses may lead to the loss of the endogenous antioxidant protection. Lower doses of appropriate antioxidants given later in pregnancy may prevent conditions related to oxidative stress such as PPCM and pre-eclampsia. Further studies are required to test this hypothesis. An increase in oxidative stress during pregnancy can be characterised by enhanced lipid peroxidation and the circulation of lipid hydroperoxides and MDA (measured using the TBARS assay)<sup>28</sup>.

In our study, the HC third trimester pregnant women did have a marginally higher MDA concentration than the HC postpartum women. This is likely due to the residual of the increase in the first and second trimesters. When the pregnancy is advanced, disruption in this oxidative balance can lead to an incorrect activation of the inflammatory cascade which produces harmful effects, including premature labour and complications such as pre eclampsia<sup>28</sup>. There was no statistically significant difference between the pregnant PPCM compared to the CD and HC women however, the means were marginally higher which may cause the PPCM and CD women to be more at risk for pre-eclampsia, frequently associated with PPCM<sup>21</sup>. The pregnant PPCM group may not be significantly higher due to the variations in pregnancy and the differences within the PPCM group of women at higher risk for reoccurrence.

In light of the increased oxidative stress in the PPCM, an ORAC assay was performed on the remaining plasma samples. There was no observable difference in the antioxidant capacity of the samples, as determined by the ORAC assay. These data, however, do not exclude a decrease in total antioxidant capacity as the ORAC only measures the vitamin E equivalent antioxidant capacity. The antioxidant capacity in the samples degrade over time and with freeze-thaw cycles. Therefore, only samples which had not been used before could be used for analysis.

#### **4.4 The mean EDPS score is elevated in postpartum PPCM women and pregnant CD women compared to HC.**

To screen for possible depression, the EPDS was administered to the pregnant and postpartum HC, CD and PPCM women. The EPDS is a validated 10 item questionnaire used for screening for a probable diagnosis of depression both pre- and postpartum<sup>85 82</sup>. Depression is known to be a risk factor for the development of cardiovascular disease, as well as a predictor of poor prognosis following a cardiac event<sup>96</sup>. The mechanism by which depression is thought to contribute is through an increase in oxidative stress, as well as inflammation<sup>99</sup>. Depression has previously been shown to increase inflammatory markers such as CRP, TNF- $\alpha$  and IL6, that have all been associated with an increased risk for cardiovascular disease<sup>99</sup>. The psychosocial risk factors for maternal and postpartum depression include past history of mental illness, mental disturbance during pregnancy, family history of depression, low socioeconomic status and poor interpersonal relationships<sup>87</sup>. Risk factors for depression during and following maternity include low education, psychosocial stressors and if previous history of depression were trimester dependant<sup>88</sup>. In developed countries, studies have shown that the prevalence of postpartum depression is around 10-15% - in developing regions this figure is 2-3 fold higher<sup>82</sup>. Two separate studies performed in South Africa found a prevalence of 32% and 39% of possible depression in healthy postpartum women, using the EDPS scale<sup>84 85</sup>.

The recommended cut-off point for EDPS score is 10 for possible depression and 20 for possible severe depression. In our study, similar to the literature, a third of the pregnant and postpartum HC women, and two-thirds of both CD and PPCM women, had a EDPS score above 10. Although the EDPS scores of the postpartum PPCM women are elevated compared to the HC women, they are not significantly elevated compared to the CD group. The average score of the PPCM group is higher, which possibly indicates more frequent or severe depression. A previous study has suggested that depression and anxiety in early pregnancy are a risk factor for pre eclampsia later in pregnancy<sup>103</sup>. A similar pattern may be found for PPCM, however it is out of the scope of the present study. The PPCM women may have been depressed before the onset of PPCM, or they may have developed the depression subsequent to the condition. The CD women displayed a higher depression score during pregnancy. As a large number are due to congenital heart disease, their heart condition may affect their lives in such a way that leads to depression. A possible explanation for the increase in the postpartum PPCM women is that previous depression contributed to the development of PPCM, and the development of PPCM worsened their depression. The EDPS scale is a self-report scale and individual differences in the interpretation of their own feelings may alter the results. The EDPS is

also only a screening tool - an elevated score warrants further investigation by a qualified mental health practitioner. During the questionnaire interview, it was noted that many of the CD and PPCM women reported feeling anxiety about dying from their condition. The high scores were often due to fear and anxiety, as well as sleep-based questions, rather than the mood-specific questions. This may indicate that the scale may be measuring depression itself, but also anxiety disorders. The scale has been validated previously in a South African population. During the interviews I found that some women had difficulty understanding. In particular, the first question, " Are you still able to laugh and see the funny side of things". The questions often had to be explained to the participants. I would recommend the scale be reworded and validated for the South African population, particularly if it is to be in studies where the questionnaire is self-administered.

Peripartum depression is disabling for women and is most common during the childbearing years<sup>81</sup>. Appropriate screening tools should be implemented, especially in developing countries where the prevalence is higher. The mental and physiological changes of the depressive women may potentially negatively affect the course of the disease<sup>96</sup>. The depressed PPCM and CD women may not adhere to treatment and their condition may worsen or, in the case of PPCM, the heart may not normalise. Previous studies have shown that depressed postpartum women also had lower levels of prolactin and that breastfeeding women have lower EDPS scores<sup>93</sup>. Bromocriptine, an inhibitor of prolactin release from the pituitary gland, is now commonly used to treat PPCM<sup>11</sup>. The PPCM women are recommended to stop breastfeeding, which may have contributed to the elevated EPDS score.

#### **4.5 The postnatal PPCM women report lower sleep efficacy, fragmented sleep and a greater sleep onset delay**

The sleeping patterns in pregnant and postpartum women are disturbed, compared to non pregnant women. Pregnant women also wake up more frequently as the foetus grows and these women often suffer nocturia and difficulty in sleeping comfortably<sup>54</sup>. Oestrogen and progesterone are known modulators of the amplitude and phase of the circadian rhythm<sup>54</sup>. It is possible that changes in these hormones during pregnancy may alter the rhythm in these women, predisposing them to mood alterations, as well as sleep disruption<sup>54</sup>. Melatonin, as well as the circadian sleep rhythm, have been demonstrated to be disturbed in the postpartum period<sup>47</sup>. Postpartum women often wake up more frequently to care for their infant<sup>47</sup>. Humans can be classified as either "evening people" or "morning people", morning people, tend to be more fatigued at night and rise earlier<sup>51</sup>. "Evening people" tend to be more active at night and rise later in the morning, with more fatigue<sup>51</sup>. Data suggests that these differences are due to individual differences in the timing and onset of melatonin release<sup>51</sup>. Circadian rhythm disturbance is also a major feature of mood disturbances<sup>54</sup>.

Even though the postpartum PPCM women tried to sleep at the same time as the HC and CD women, it took them longer to fall asleep. This may be due to the symptoms of PPCM or other factors, such as a sleep disorder or depression, Orthopnoea is a common symptom and could contribute to difficulty falling asleep. All the pregnant and postpartum women reported an increase in sleep disruption and sleep fragmentation, compared to before pregnancy. The postpartum PPCM women reported a higher frequency than the postpartum CM and HC women in nocturnal waking, longer time between sleep and longer waking time compared to the CD and HC women. This may again be due to the symptoms of PPCM, such as palpitations, or may have another underlying cause. The PPCM women also reported less energy following waking, compared to the HC group. The symptoms of PPCM include fatigue and may contribute to the reports of fatigue, compared to the HC group.

Circadian rhythm disturbance is also a major feature of mood disturbances<sup>54</sup>. Sleep disruption is a common symptom of both postpartum depression and major depression. Sleep disruption may also lead to a lower mood. Whether the PPCM symptoms caused the sleep disruption, which may lead to the higher depression scores, will need to be further investigated.

#### **4.6 The serum cortisol concentrations are elevated in pregnant and postpartum women with PPCM**

The steroid hormone cortisol, is released in response to stress and is commonly elevated in psychiatric conditions such as anxiety and depression<sup>89</sup>. There has been previous reports noting an increase in blood cortisol during pregnancy, with a 2- to 3-fold increase in the final trimester<sup>91</sup>. In healthy women the cortisol levels return to baseline shortly following birth.

There was a significant increase in serum cortisol in the postpartum women with PPCM. In the present study cortisol concentrations in pregnant and postpartum HC, CD and PPCM women was quantified. The mean serum cortisol in healthy pregnant women was almost 2-fold higher than in healthy postpartum women. This is in keeping with previous reports of the increase in cortisol during pregnancy. There was no significant difference in serum cortisol during pregnancy between the groups.

There was a positive correlation between cortisol levels and EDPS scores in the postpartum women. There was no correlation between the EDPS score and cortisol level during pregnancy and this may be due to the natural disruption of cortisol and other pregnancy related factors. Alternately, other factors during pregnancy, such as anxiety, which is not measured by EDPS, may affect the correlation. There was a mildly positive correlation between cortisol and EDPS scores during the postpartum period.

This suggests an increase in stress or a psychiatric condition such as depression. This correlated to an increase in the EDPS score, suggesting that postpartum women with PPCM are more depressed than controls. This, however, cannot determine whether the increase in stress/anxiety/depression contributes towards the development of PPCM or whether the increase in stress is due to the stress of being diagnosed with a potentially life-threatening condition. There were many women in the study who reported a fear of dying. There was also an increase, however not significantly, in the CD group compared to the HC group. This may be lower than the PPCM group due to the variation in the severity of their conditions. There was no significant difference in the postpartum cortisol levels of the CD women compared to the PPCM women. This may suggest that the increase in stress is due to the condition and not a contributor. The only method to determine whether stress and an increase in cortisol contributes to PPCM would be a long-term study, such as a longitudinal study.

#### 4.7 Salivary melatonin is elevated in the postpartum PPCM women

Melatonin is renowned as a "sleep hormone" and acts as a time-giver, essential to both the timing and onset of circadian rhythm and reproduction<sup>46</sup>. Human average melatonin levels are lowest during daytime hours and peak around 2-4am and decrease towards the morning hours<sup>50</sup>. Melatonin is also a powerful natural antioxidant, able to easily reach cellular and sub cellular compartments, due to its small size and amphiphilic nature<sup>47</sup>. Melatonin has previously been shown to activate the cardioprotective SAFE pathway that involves the activation of TNF and STAT3<sup>60</sup>. Preliminary studies suggest melatonin levels are lower in subjects with coronary artery disease<sup>45</sup>. Evidence in the literature indicate that melatonin is a potential therapy, either alone or in combination, in conditions with oxidative stress<sup>32</sup>. Melatonin has multiple pleiotrophic effects on the heart, including anti-inflammatory, antioxidant and antihypertensive effects<sup>58</sup>. In women with severe pre-eclampsia, the serum melatonin levels have been shown to be significantly lower<sup>46</sup>. Pre-eclampsia is another cardiovascular disease associated with pregnancy. Melatonin was hypothesised to show a similar decrease in PPCM.

The nocturnal (2am) and daytime (baseline) salivary melatonin concentration was determined in the pregnant and postnatal HC, CD and PPCM women by ELISA. Unlike the women with severe pre-eclampsia, we were surprised to find that the postpartum PPCM women demonstrated significantly higher nocturnal melatonin, compared to the CD and HC women. The daytime melatonin levels were also slightly elevated, but not significantly. Melatonin concentrations during pregnancy have been shown to be suppressed in the first trimester with the increase in oxidative stress and, thereafter, rise and return to baseline postpartum<sup>46</sup>. Depressed pregnant and postpartum women have been shown to have different patterns in melatonin concentrations. Depressed pregnant women have previously been shown to have lower melatonin levels and depressed postpartum women higher melatonin levels than controls<sup>105 54</sup>. The postnatal PPCM women demonstrated higher cortisol and EDPS scores postpartum, which may explain the increase in nocturnal melatonin. The PPCM women also reported more sleep fragmentation and sleep efficiency, logically this would indicate lower melatonin concentrations. A previous study found the inverse, that lower sleep quality was correlated with higher melatonin concentrations<sup>104</sup>. This has been suggested to be due to the natural defence response to increase in oxidative stress caused by change in diet or lifestyle in response to mental stress<sup>104</sup>. Chronic poor sleep quality is likely to increase the melatonin concentration in as a defence mechanism against the sleep disruption<sup>104</sup>.

Melatonin has also been shown to suppress prolactin secretion in animal models (rats and ewes)<sup>55 54</sup>. This suggests that melatonin and prolactin may also have a similar relationship in humans. If melatonin supplementation has the effect of inhibiting prolactin, possibly the inhibition of prolactin may raise the levels of melatonin. Bromocriptine, an inhibitor of prolactin secretion, has been suggested as a potential treatment for PPCM and 7 of the 9 postnatal PPCM women in this study received treatment with the drug. The increased levels of melatonin may act as a natural endogenous mechanism to limit the progression of the disease. It would be relevant to study whether bromocriptine suppresses prolactin secretion, in part, by raising melatonin.

There are individual differences in the timing and onset of the release of melatonin<sup>51</sup>. The nocturnal melatonin was only sampled at 2am. A full 24-hour sampling every hour would be required to compare the full melatonin rhythm. Hence, the observed increase may be due to a shift in the release in melatonin levels in the PPCM group, with an earlier release leading to a higher concentration. Melatonin should be measured under controlled light conditions as even dim light can suppress melatonin release. Variations may therefore be observed in women who did not fully adhere to the written instructions<sup>50</sup>.

Although the results suggest an increase in melatonin in the PPCM group, melatonin should not be excluded as a potential prevention or treatment in cardiovascular disease such as PPCM. Previous studies have failed to show a benefit using supplementation with classical antioxidant vitamins A, E and C<sup>32</sup>. Melatonin is a powerful scavenger of both RNS and ROS, including those formed from peroxynitrite, and blocks transcriptional factors, which induce pro inflammatory cytokines, as well as presenting additional benefits independent of its antioxidant capacity<sup>32</sup>. The use of exogenous melatonin supplementation has previously been reported to induce drowsiness and sleep and has been suggested to be beneficial in alleviating sleep disturbances and nocturnal awakenings<sup>52</sup>. There have been other beneficial effects of melatonin supplementation during pregnancy, such as the prevention of pre-eclampsia and protecting the foetus against neurological damage<sup>46</sup>.

#### **4.8 Limitations to study**

The samples size of the study was relatively small and collected from a single centre (CDMC). The study used two questionnaires to measure depression scores and sleep disruption. Questionnaires are notoriously subjective and participants tend towards more socially desirable answers, as well as understating or exaggeration of answers. The study was also limited by the willingness of participants to collect their own saliva samples and return them the following day. While the utmost care was taken to correctly instruct the patients to avoid bright light and collect the samples at the correct time, this cannot be guaranteed. Ideally the saliva samples should have been sampled hourly, to obtain a detailed pattern of the release of melatonin. Due to most of the PPCM women only being diagnosed in the postpartum phase, it is impossible to determine whether the sleep disruption, depression, melatonin and cortisol levels are a result of or a contributing factor in the development of PPCM. Also most of the postnatal PPCM women received bromocriptine, which could not be controlled. This may affect the oxidative stress assays, as well as the depression score results. Furthermore, bromocriptine may likely have raised the melatonin levels in the postnatal PPCM women. The number of pregnancies of the patients was also not reported and multiple pregnancies may affect oxidative stress and depression levels. While the blood samples were handled carefully, there was loss of samples due to haemolysis of the blood being excluded from the oxidative stress assays. The assays were also limited by the amount of blood drawn from the participant.

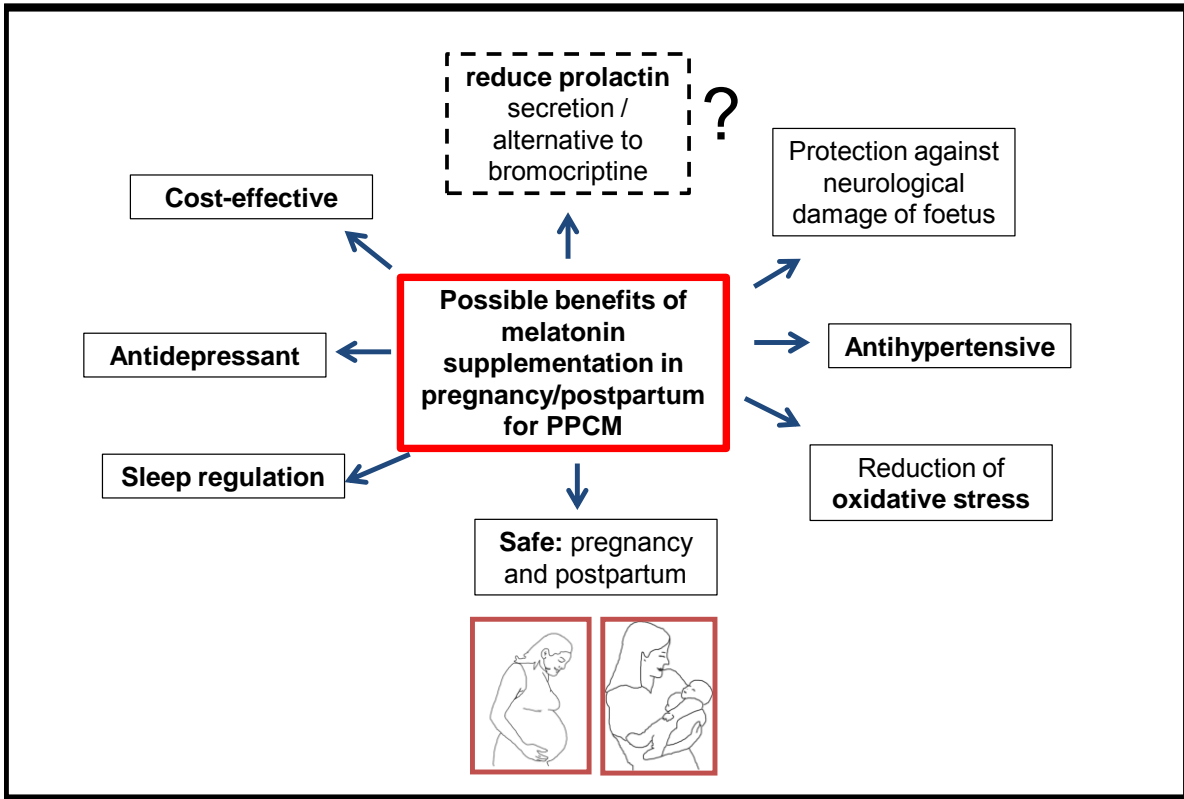
## 4.9 Conclusion

The postpartum women diagnosed with PPCM showed higher concentrations of BNP and an increase in oxidative stress in the form of lipid peroxidation, corresponding with previous findings. The PPCM group had higher EDPS scores with higher serum cortisol, as well as signs of sleep disruption. In the future, a possible study could be widespread screening of pregnant women, at multiple centres, during pregnancy to determine whether they are more at risk for the development of PPCM or other conditions. Conditions such as peripartum depression are more common in developing countries, such as South Africa, and there is a need for more efficient screening methods, as well as treatment, as these conditions have a detrimental effect on the health of the population.

Unlike the study on severe pre-eclampsia, also thought to be the result of an increase in oxidative stress where the melatonin levels were lowered, the postpartum PPCM group demonstrated higher melatonin levels. Further studies with a larger sample size are needed to determine whether this increase is due to Bromocriptine treatment, as a possible defence mechanism in response to the increase in oxidative stress or a shift in melatonin in secretion.

In developing countries, there is a higher prevalence of PPCM and a high mortality rate, with approximately 1 in 5 women dying, even on treatment. It is therefore important that cost-effective, safe and effective alternative treatments or prevention strategies be found. The potential benefits of melatonin use are outlined in **Figure 43**. If melatonin proves to be an important mediator of bromocriptine-induced suppression of prolactin, the use of low dose melatonin supplementation may prove to be a more desirable alternative. Bromocriptine cannot be used during pregnancy, while the use of low doses of melatonin has additional benefits during pregnancy. Bromocriptine also has undesirable side-effects, such as the full suppression of breastfeeding, as well as the reported thrombotic risks. Supplementation with exogenous melatonin may allow for the safe reduction in prolactin and a reduction in the oxidative stress, which has been shown to contribute to the development of PPCM. Melatonin has the additional benefits, to regulate sleeping pattern and antidepressant effects, common symptoms observed in the women with PPCM. Melatonin has previously been shown to be antihypertensive, which may be beneficial due to the high co morbidity with pre-eclampsia. There should be caution with the timing of melatonin as supplementation, as it may not be beneficial in the first trimester where oxidative stress has necessary physiological functions. Further studies are needed to prove the hypothesis that melatonin may be a useful

treatment and even, possibly, prevent PPCM. If these studies prove beneficial, melatonin may be a simple, cheap and effective therapy for the treatment/prevention for PPCM.



**Figure 43.** Potential benefits of melatonin supplementation in the treatment of PPCM.

## REFERENCES

1. Brar SS, Khan SS, Sandhu GK, et al. Incidence , Mortality , and Racial Differences in Peripartum Cardiomyopathy. *The American Journal of Cardiology*. 2007;323:10–12.
2. Sliwa K, Fett J, Elkayam U. Peripartum cardiomyopathy. *Lancet*. 2006;368:1–7.
3. Anderson JL, Horne BD. Birthing the Genetics of Peripartum Cardiomyopathy. *Circulation*. 2012;121:2157–2159.
4. Sliwa K, Förster O, Libhaber E, et al. Peripartum cardiomyopathy: inflammatory markers as predictors of outcome in 100 prospectively studied patients. *European heart journal*. 2006;27:441–6.
5. Sliwa K, Hilfiker-kleiner D, Petrie MC, et al. Current state of knowledge on aetiology , diagnosis , management , and therapy of peripartum cardiomyopathy : a position statement from the Heart Failure Association of the European Society of Cardiology Working Group on peripartum cardiomyopathy. *European Journal of Heart Failure*. 2010;12:767–778.
6. Blauwet LA, Cooper LT. Diagnosis and management of peripartum cardiomyopathy. *Heart*. 2011;97:1970–1981.
7. Clapp C, Thebault S, Martínez de la Escalera G. Hormones and postpartum cardiomyopathy. *Trends in endocrinology and metabolism*. 2007;18:329–30.
8. Leinwand LA. Molecular Events Underlying Pregnancy- Induced Cardiomyopathy. *Cell*. 2007;1:437–438.
9. Sliwa K, Mayosi BM. Recent advances in the epidemiology , pathogenesis and prognosis of acute heart failure and cardiomyopathy in Africa. *British Medical Journal*. 2013;18:1–6.
10. Patten IS, Rana S, Shahul S, et al. Cardiac Angiogenic Imbalance Leads to Peri-partum Cardiomyopathy. *Nature*. 2012;485:333–338.
11. Hilfiker-kleiner D, Schieffer E, Meyer GP, Podewski E. Postpartum Cardiomyopathy. *Medicine*. 2008;105:751–756.
12. Cowie MR, Jourdain P, Maisel A, et al. Clinical applications of B-type natriuretic peptide ( BNP ) testing. *European heart journal*. 2003;24:1710–1718.
13. Walenta K, Schwarz V, Schirmer SH, et al. Circulating microparticles as indicators of peripartum cardiomyopathy. *European Heart Journal*. 2012;33:1469–1479.
14. Spaendonck-zwarts KY Van, Tintelen JP Van, Veldhuisen DJ Van, et al. Peripartum Cardiomyopathy as a Part of Familial. *Circulation*. 2010;20:2169–2175.
15. Morales A, Painter T, Li R, Siegfried JD, Li D, Norton N. Mutations in 6 genes identified in patients with peripartum cardiomyopathy. *Journal of the American College of Cardiology*. 2010;55:122–131.

16. Abbas A., Lichtman A. *Inate Immunology (3rd Edition)*.; 2009:3.
17. Forster O, Hilfiker-Kleiner D, Denise Ansari A, Sundstrom B, et al. Reversal of IFN- $\gamma$ , oxLDL and prolactin serum levels correlate with clinical improvement in patients with peripartum cardiomyopathy. *European Journal of Heart Failure*. 2008;10:861–868.
18. Hilfiker-kleiner D, Hilfiker A, Drexler H. Many good reasons to have STAT3 in the heart. *Pharmacology & Therapeutics*. 2005;107:131–137.
19. Hilfiker-Kleiner D, Kaminski K, Podewski E, et al. A cathepsin D-cleaved 16 kDa form of prolactin mediates postpartum cardiomyopathy. *Cell*. 2007;128:589–600.
20. Forster O, Hilfiker-Kleiner D., Ansari AA, et al. Reversal of IFN- $\gamma$ , oxLDL and prolactin serum levels correlate with.pdf. *The European journal of heart failure*. 2008;10:861–868.
21. Reuwer AQ, Reuwer PJHM, Post JA Van Der, et al. Prolactin fragmentation by trophoblastic matrix metalloproteinases as a possible contributor to peripartum cardiomyopathy and pre-eclampsia. *Medical Hypotheses*. 2010;74:348–352.
22. Fett JD, Ansari AA, Sundstrom JB, Jr GFC. Peripartum cardiomyopathy : a selenium disconnection and an autoimmune connection. *Public Health*. 2002;86:311–316.
23. Haghikia A, Podewski E, Libhaber E, et al. Phenotyping and outcome on contemporary management in a German cohort of patients with peripartum cardiomyopathy. *Basic research in Cardiology*. 2013;108:1–13.
24. Goland S, Modi K, Bitar F, et al. Clinical Profile and Predictors of Complications in Peripartum Cardiomyopathy. *Journal of Cardiac Failure*. 2009;6:1–6.
25. Elkayam U, Tummala P, Rao K, et al. Maternal and fetal outcomes of subsequent pregnancies in women with peripartum cardiomyopathy. *The New England Journal of Medicine*. 2001;344:1567–1571.
26. Sliwa K, Blauwet L, Tibazarwa K, et al. Evaluation of bromocriptine in the treatment of acute severe peripartum cardiomyopathy: a proof-of-concept pilot study. *Circulation*. 2010;121:1465–73.
27. Halkein J, Tabruyn SP, Ricke-hoch M, et al. MicroRNA-146a is a therapeutic target and biomarker for peripartum cardiomyopathy. *The Journal of Clinical Investigation*. 2013;123:2143–2154.
28. Biondi C, Pavan B, Lunghi L, Fiorini S, Vesce F. The Role and Modulation of the Oxidative Balance in Pregnancy. *Current Pharmaceutical Design*. 2005;2:2075–2089.
29. Berger M. Can oxidative damage be treated nutritionally ? *Clinical Nutrition*. 2005;24:172–183.
30. Hensley K, Robinson K, Gabbita S, Salsman S, Floyd R. Reactive oxygen species, cell signalling, and cell injury. *Free radical biology medicine*. 200AD;28:1456–62.

31. Virag L, Szabo S, Gergely P, Szabo C. Peroxynitrite-induced cytotoxicity: mechanism and opportunities for intervention. *Toxicology letters*. 2003;140:113–124.
32. Korkmaz A, Reiter RJ, Topal T, Manchester LC, Oter S, Tan D. Melatonin : An Established Antioxidant Worthy of Use in Clinical Trials. *Molecular medicine*. 2009;5:3–10.
33. Poston L, Rajmakers MTM. Trophoblast oxidative stress, antioxidants and pregnancy outcome--a review. *Placenta*. 2004;25:72–78.
34. Lander H, Milbank A, Tauras J, Hajjar D, Hempstead B, Schwartz G. Redox regulation of cell signalling. *Nature*. 1996;81:380–381.
35. Blomhoff R. Dietary antioxidants and cardiovascular disease. *Current Opinion in Lipidology*. 2005;16:47–54.
36. Romero R, Gomez R, Grezzi F, Yoon B, Mazor M, Eddwin S. A foetal systemic inflammatory response is followed by the spontaneous onset of preterm parturition. *Journal of Obstetrics and Gynaecology Gynecol*. 1998;179:186–93.
37. Buhimschi I, Yallampalli C, Dong Y-L, Garfield R. Involvement of a nitric oxide- guanosine monophosphate pathway in control of human uterine contractility during pregnancy. *Journal of Obstetrics and Gynaecology*. 1995;172:1577–84.
38. Casanueva E, Viteri F. Iron and Oxidative Stress in Pregnancy. *World Health*. 2003;133:1700–1708.
39. Sharma JB, Sharma A, Bahadur A, Vimala N. Oxidative stress markers and antioxidant levels in normal pregnancy and pre-eclampsia. *Seminars in Nephrology*. 2006;5:23–27.
40. Ceconi C, Boraso A, Cargnoni A, Ferrari R. Oxidative stress in cardiovascular disease: myth or fact? *Archives of Biochemistry and Biophysics*. 2003;420:217–221.
41. Tengattini S, Russel J, Tan D, Pilar M, Rodella LF. Cardiovascular diseases : protective effects of melatonin. *Journal of Pineal Research*. 2008;44:16–25.
42. Tsutsui H, Kinugawa S, Matsushima S. Mitochondrial oxidative stress and dysfunction in myocardial remodelling. *Cardiovascular Research*. 2009;10:449–456.
43. Poston L, Briley AL, Seed P, Kelly F, Shennan A. Vitamin C and E in pregnant women at risk for pre-eclampsia (VIP trial): randomised placebo controlled trial. *Lancet*. 2006;367:1145–54.
44. Reiter RJ, Tan D. Melatonin : a novel protective agent against oxidative injury of the ischemic / reperfused heart. *Cardiovascular Research*. 2003;58:10–19.
45. Claustrat B, Brun J, Chazot G. The basic physiology and pathophysiology of melatonin. *Physiological review*. 2005;9:11–24.
46. Nakamura Y, Tamura H, Kashida S, Takayama H, Yamagata Y. Changes of serum melatonin level and its relationship to feto-placental unit during pregnancy. *Journal of pineal research*. 2001;30:29–33.

47. Thomas KA, Burr RL. Melatonin Level and Pattern in Postpartum Versus Nonpregnant Nulliparous Women. *JOGNN*. 2006;5:608–615.
48. Reiter RJ, Paredes SD, Manchester LC, Tan D. Reducing oxidative / nitrosative stress : a newly-discovered genre for melatonin. *Critical Reviews in Biochemistry and Molecular Biology*. 2009;44:175–200.
49. Shilo L, Sabbah H, Hadari R, et al. The effects of coffee consumption on sleep and melatonin secretion. *Brain Research Brain Research Reviews*. 2002;3:271–273.
50. Voultzios A. Circadian Rhythm of Free Melatonin in Human Plasma. *Endocrinology And Metabolism*. 2000;83:1013–1015.
51. Liu X, Uchiyama M, Shibui K, et al. Diurnal preference , sleep habits , circadian sleep propensity and melatonin rhythm in healthy human subjects. *Neuroscience Letters*. 2000;280:199–202.
52. Brzezinski A, Vangel MG, Wurtman RJ, et al. Effects of exogenous melatonin on sleep: a meta-analysis. *Sleep medicine reviews*. 2005;9:41–50.
53. Higuchi S, Motohashi Y, Ishibashi K, Maeda T. Influence of eye colors of Caucasians and Asians on suppression of melatonin secretion by light. *American Journal Of Physiology*. 2013;7:121–122.
54. Parry BL, Meliska CJ, Ph D, et al. Plasma Melatonin Circadian Rhythms: Low in Pregnant, Elevated in Postpartum, Depressed Women, and Phase- Advanced in Pregnant Women with Personal or Family Histories of Depression. *Am J Psychiatry*. 2008;165(:1551–1558).
55. Juszczak M, Stempnaik B. The effect of melatonin on suckling-induced oxytocin and prolactin release in the rat. *Brain Research Bulletin*. 1997;44:253–258.
56. Molik E, Misztal T, Romanowicz K, Zieba DA. The effects of melatonin on prolactin and growth hormone secretion in ewes under different photoperiods , during the early post partum period. *Small Ruminant Research*. 2010;94:137–141.
57. Mediavilla MD, Tan DX, Reiter RJ. Clinical Uses of Melatonin : Evaluation of Human Trials. *Current Medicinal Chemistry*. 2010;17:2070–2095.
58. Dominguez-rodriguez A, Abreu-gonzalez P, Reiter RJ. Melatonin and Cardiovascular Disease : Myth or Reality ? *Revista Española de Cardiología (English Edition)*. 2012:2–5.
59. Lecour S, James RW. When are pro-inflammatory cytokines SAFE in heart failure ? *European Heart Journal*. 2011;6:680–685.
60. Lamont KT, Somers S, Lacerda L, Opie LH, Lecour S. Is red wine a SAFE sip away from cardioprotection? Mechanisms involved in resveratrol- and melatonin-induced cardioprotection. *Journal of pineal research*. 2011;50:374–80.
61. Moore-Ede M, Sulzman FM, Fuller CA. The Clocks That Time Us. *Harvard University Press*. 1982.

62. Rea MS, Bierman A, Figueiro MG, Bullough JD. A new approach to understanding the impact of circadian disruption on human health. *Journal of Circadian Rhythms*. 2008;14:1–14.
63. Silber MH, Ch MBB, Ancoli-israel S, et al. The Visual Scoring of Sleep in Adults. *Journal of clinical sleep medicine*. 2007;3:121–125.
64. Czeisler C, Weitzman E, Moore-Ede M, Zimmerman J, Knauer R. Human sleep: its duration and organization depend on its circadian phase. *Science*. 1980;210:1264–1267.
65. Rechtschaffen A, Hauri P, Zeitlin ANDM. Sleep stages. *Public Health*. 1966;1:927–942.
66. Rajaratnam SMW, Arendt J. Health in a 24-h society. *The Lancet*. 2001;358:999–1005.
67. Backhaus J, Junghanns K, Broocks A, Riemann D, Hohagen F. Test – retest reliability and validity of the Pittsburgh Sleep Quality Index in primary insomnia. *Journal of Psychosomatic Research*. 2002;53:737–740.
68. Gillberg M, Keckland G, ALexson J, Akerstet T. The effects of a short daytime nap after a restricted nights sleep. *American association and sleep research society*. 1996;19:570–575.
69. Pilcher JJ, Ginter DR, Sardowsky BR. Sleep quality vs sleep quantity : relationships between measures of health and well being as well as sleepyness in colledge students. *Journal of psychosomatic research*. 1997;42:583–596.
70. Okun ML, Coussons-Read ME. Sleep disruption during pregnancy : How does it influence serum cytokines ? *Journal of Reproductive Immunology*. 2007;73:158–165.
71. Maurer EL, Lo AM, Parry BL, Marti LF, Sorenson D, Meliska CJ. Sleep , rhythms and women ' s mood . Part I . Menstrual cycle , pregnancy and postpartum. *Sleep medicine*. 2006;6:129–144.
72. Montgomery-Downs HE, Insana SP, Clegg-Kraynok MM, Mancic LM. Normative longitudinal maternal sleep: the first 4 postpartum months. *American journal of obstetrics and gynecology*. 2010;203:465–467.
73. Scheer FAJL, Hilton MF, Mantzoros CS, Shea SA. Adverse metabolic and cardiovascular consequences of circadian misalignment. *PNAS*. 2009;106:1–6.
74. Irwin MR, Wang M, Ribeiro D, et al. Sleep Loss Activates Cellular Inflammatory Signaling. *Biological Psychiatry*. 2009;64:538–540.
75. Shamsuzzaman ASM, Winnicki M, Lanfranchi P, Wolk R, Accurso V, Somers VK. Elevated C-Reactive Protein in Patients With Obstructive Sleep Apnea. *Circulation*. 2002;105:2462–2464.
76. Redeker NS, Hilkert R. Sleep and Quality of Life in Stable Heart Failure. *Journal of Cardiac failure*. 2005;11:700–704.
77. Redeker N, Ruggiero J, Hedges C. Sleep is related to physical function and emotional wellbeing after cardiac surgery. *Nurse research*. 2004;53:154–62.

78. Remick R a. Diagnosis and management of depression in primary care: a clinical update and review. *CMAJ : Canadian Medical Association journal*. 2002;167:1253–60.
79. Kendler KS, Gardner CO. Boundaries of major depression: an evaluation of DSM-IV criteria. *The American journal of psychiatry*. 1998;155:172–7.
80. Farmer A, McGuffin P. The classification of the depressions: contemporary confusion revisited. *British Journal of Psychiatry*. 1989;155:437–443.
81. Hara MWO. Postpartum Depression : What We Know. *Journal of clinical psychology*. 2009;65:1258–1270.
82. Lumley J, Austin M. What interventions may reduce postpartum depression. *Current Opinion in Obstetrics and Gynecology*. 2001;13:605–611.
83. Adewuya AO. The maternity blues in Western Nigerian women : Prevalence and risk factors. *Psychiatry: Interpersonal and Biological Processes*. 2005;193:1522–1525.
84. Honikman S, Van Heyningen T, Field S, Baron E, Tomlinson M. Stepped care for maternal mental health: a case study of the perinatal mental health project in South Africa. *PLoS medicine*. 2012;9(5):1–6.
85. Hartley M, Tomlinson M, Greco E, et al. Depressed mood in pregnancy : Prevalence and correlates in two Cape Town peri-urban settlements. *Reproductive Health*. 2011;8(1): 1742-4755
86. Olley BO, Seedat S, Nei DG, Stein DJ. Predictors of Major Depression in Recently Diagnosed Patients with HIV/AIDS in South Africa. *Aids Patient Care*. 2004;18(8).481-487
87. Verkerk GJM, Pop VJM, Son MJM Van, Heck GL Van. Prediction of depression in the postpartum period : a longitudinal follow-up study in high-risk and low-risk women. *Journal of Affective Disorders*. 2003;77:159–166.
88. Bunevicius R, Kusminskas L, Bunevicius A, Nadisauskiene RJ, Jureniene K, Pop VJM. Psychosocial risk factors for depression during pregnancy. *Acta Obstetrica et Gynecologica*. 2009;88:599–605.
89. Evans LM, Myers MM, Monk C. Pregnant women’s cortisol is elevated with anxiety and depression — but only when comorbid. *Health (San Francisco)*. 2008;5:239–248.
90. Selmaoui B, Touitou Y. Reproducibility of the circadian rhythms of serum cortisol and melatonin in healthy subjects : a study of three different 24-h cycles over six weeks. *Life Sciences*. 2003;73:3339–3349.
91. Meulenberg PMM, Hofman JA. Differences between Concentrations of Salivary Cortisol and Cortisone and of Free Cortisol and Cortisone in Plasma during Pregnancy and Postpartum. *Clinical Chemistry*. 1990;36:70–75.
92. In HE. Hormonal Changes in the Postpartum and Implications for Postpartum Depression. *Psychosomatics*. 1998;11:93–101.

93. Abou-saleh MT, Ghubash R, Karim L, Krymski M, Bhai I. Hormonal aspects of postpartum depression. *Science*. 1998;23:465–475.
94. Buckwalter JG, Stanczyk FZ, McCleary CA, et al. Pregnancy, the postpartum, and steroid hormones: effects on cognition and mood. *Psychoneuroendocrinology*. 1999;24:69–84.
95. Suls J, Bunde J. Anger, Anxiety, and Depression as Risk Factors for Cardiovascular Disease: The Problems and Implications of Overlapping Affective Dispositions. *Psychological Bulletin*. 2005;131:260–300.
96. Joynt KE, Whellan DJ, Connor CMO. Depression and Cardiovascular Disease: Mechanisms of Interaction. *Biological Psychiatry*. 2003;54:248–261.
97. Anda R, Williamson D, Jones D, et al. Depressed Affect, Hopelessness, and the Risk of Ischemic Heart Disease in a Cohort of U.S. Adults. *Epidemiology*. 1993;4:285–294.
98. Newcomer JW, Hennekens CH. Severe Mental Illness and Risk of Cardiovascular Disease. *American medical association*. 2013; 1794–1797.
99. Vaccarino V, Johnson BD, Sheps DS, et al. Depression, inflammation, and incident cardiovascular disease in women with suspected coronary ischemia: the National Heart, Lung, and Blood Institute-sponsored WISE study. *Journal of the American College of Cardiology*. 2007;50:2044–50.
100. Kamarck W, Stewart J, Rand K, Muldoon M. A prospective study of the directionality of the depression inflammation relationship. *Brain behaviour immunology*. 2009;23:936–944.
101. Hilfiker-Kleiner D, Sliwa K, Drexler H. Peripartum Cardiomyopathy: Recent Insights in its Pathophysiology. *Trends in Cardiovascular medicine*. 2008;18:174–179.
102. Villar J, Purwar M, Meriandi M, et al. World Health Organisation multicentre randomised trial of supplementation with vitamins C and E among pregnant women at high risk for pre-eclampsia in populations of low nutritional status from developing countries. *Journal of Obstetrics and Gynaecology*. 2009;116:780–788.
103. Kurki T, Hiilesmaa V, Raitasalo R, Mattila H, Ylikorkala O. Depression and Anxiety in Early Pregnancy and Risk for Preeclampsia. *Obstetrics & Gynecology*. 2000;95:487–490.
104. Taki Y, Iida T, Nakayama M, Nakagami Y. Sleep quality and mental stress influence salivary melatonin concentrations. *Journal of analytical Bio-science*. 2012;35:234–240.
105. Parry BL, Meliska CJ, Ph D, et al. Pregnancy and Postpartum in Depressed Women and Women With Personal or Family Histories of Depression. *Psychiatry: Interpersonal and Biological Processes*. 2008:1551–1558.
106. Hall C. Essential biochemistry and physiology of NT-pro BNP. *The European journal of heart failure*. 2004;6:257–260.

107. Sawada Y, Suda M, Yokoyama H. processing of braintype natriuretic peptide are controlled by proprotein-processing endoprotease furin. *Journal of Biological Chemistry*. 1997;20:545–554.
108. McMurray JJ V, Anker SD, Auricchio A, et al. 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 Fai. *European Heart Journal*. 2012;33:1787–1847.
109. Pryor A. The antioxidant nutrients and disease do we know and what do we need to find out ? *American Journal of Clinical Nutrition*. 1991;53:391–393.
110. Jentsch A, Bachmann H, Furst P, Biesalski H. Improved analysis of malondialdehyde in human body fluids. *Free Radic Biol Med*. 1996;20:251–256.
111. Cao G, Booth SL, Sadowski J a, Prior RL. Increases in human plasma antioxidant capacity after consumption of controlled diets high in fruit and vegetables. *The American journal of clinical nutrition*. 1998;68:1081–7.
112. Kazi AM, Khalid W. Questionnaire designing and validation Introduction and Objectives : Questionnaire Development : Validity : Questionnaire Style and Appearance : *The Journal of the Pakistan Medical Association*. 2012;62:514–516.
113. Bennett J a., Riegel B, Bittner V, Nichols J. Validity and reliability of the NYHA classes for measuring research outcomes in patients with cardiac disease. *Heart & Lung: The Journal of Acute and Critical Care*. 2002;31:262–270.

## **APPENDICIES**

### **APPENDIX A**

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## APPENDIX B

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## APPENDIX C

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## APPENDIX D

### ORAC reagents

#### Phosphate buffer(0.075M pH=7.4):

- 0.75M  $K_2HPO_4 \cdot 3H_2O$  (MW= 228.23: 85.6g  $\rightarrow$ 500mL d  $H_2O$ )
- 0.75M  $Na_2H_2PO_4 \cdot H_2O$  (MW= 137.99: 51.8g  $\rightarrow$ 500mL d  $H_2O$ )

90mL  $K_2HPO_4$  +24mL  $Na_2H_2PO_4$

Make up to 900mL then pH , make up to 1L

#### Trolox:(6-OH-2,5,7,8-tetromethylchorman-2-carboxylic acid)

0.005g Trolox in 200 $\mu$ l ethanol = 100mM

1. 100 $\mu$ l + 9.9  $\mu$ l buffer =1000 $\mu$ M
2. 1mL + 9mL buffer =100 $\mu$ M (5nmol)
3. 300 $\mu$ l +300  $\mu$ l buffer =50  $\mu$ M (2.5nmol)
4. 300 $\mu$ l +300  $\mu$ l buffer =25  $\mu$ M (1.25nmol)
5. 300 $\mu$ l +300  $\mu$ l buffer =12.5  $\mu$ M (0.625nmol)
6. 300 $\mu$ l +300  $\mu$ l buffer =6.25  $\mu$ M (0.313nmol)
7. 300 $\mu$ l +300  $\mu$ l buffer =3.13  $\mu$ M (0.156nmol)
8. 300 $\mu$ l +300  $\mu$ l buffer =1.37  $\mu$ M (0.078nmol)

#### Fluorescien (3,6-dihydroxyspiro(isoberyofuran-1(3H),9(9H)-xanthen) (disodium)

- Stock 1: 0.0225g in 50mL buffer (0.0011959 mol/L)
- Stock 2: 50 $\mu$ l stock 1 in 10mL buffer 1 in 10m/L buffer (5.98 $\mu$ mol/L)
- Working solution: 320 $\mu$ l stock 2 in 20mL buffer (95.7nmol/L)

#### AAPH: (2,2 -azobis(2-andinopropane)dihydrocholride)

NB: Prepare immediately before use in phos. buffer pre washed to 37°C

- 0.087g + 980 $\mu$ l warm buffer
- (0.348g + 3920 $\mu$ l warm buffer) =32.1  $\mu$ mol per well
- 0.696 + 7840 warm buffer

