The Role of miR-29a, miR-181a, and miR-222 in Preeclamptic and Gestational Hypertensive Patients.

By

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PREFACE

The study described in this dissertation was carried out by Mrs Olive P. Khaliq and has not been submitted in any other form to another University. This study was carried out in the Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa from February 2016 to May 2017 under the supervision of Prof Irene Mackraj.

Olive P. Khaliq _____ Date _____23/08/2017_____

(216074799)

Professor Irene Mackraj

Supervisor _____ Date_____

DECLARATION

I, Mrs. Olive P. Khaliq declare as follows:

1. That the work described in this thesis has not been submitted to UKZN or other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party.

2. This thesis does not contain other person's writing, data, pictures, or other information unless specifically acknowledged as being sourced from other persons or researchers. Where other written sources have been quoted then:

Their words have been re-written but the general information attributed to them has been referenced.

Where their exact words have been used, then it has been properly referenced in the reference section.

3. Signed…… … Date……23/08/2017……

DEDICATION

I would like to dedicate this project to:

1. My mother Joyce Ellen McPherson and my father James Arthur McPherson for all the love, the support, the guidance and encouragement. I would not have made it this far without you.
2. My husband Pervaiz Khaliq and our beloved children, Hakeem Abdul Khaliq and Hammaad Khaliq.
3. My late father-in-law Mr Abdul Khaliq

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4. My research colleague **Mr Simeone Eche**, for his assistance, support, guidance and advice.
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PUBLICATION

The following manuscript has been submitted to the Molecular and Cellular and Biochemistry (MCB) Journal. (Manuscript number: MCBI-D-17-00516).

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ABBREVIATIONS AND SYMBOLS

AKT	A Threonine/Serine Protein Kinase
BMI	Body Mass Index
BREC	Biomedical Research Ethics Committee
CTBs	Cytotrophoblasts
EVTs	Extravillous Trophoblasts
enEVTs	Endovascular Extravillous Trophoblasts
GH	Gestational Hypertension
HDP	Hypertensive Disorders of Pregnancy
iEVTs	Interstitial Extravillous Trophoblasts
miRNAs	MicroRNAs
µl	Microliters
PE	Preeclampsia
PI3K	Phophatidyl-inositol-3-kinase
RT-qPCR	Real-time Quantitative Polymerase Chain Reaction
STB	Syncytiotrophoblast
sEng	Soluble Endoglin
sFlt-1	Soluble Endothelial Growth Factor Receptor-1
t-test	A type of statistical test
VEGF	Vascular Endothelial Growth Factor
≥	Greater than and equal to
≤	Less than and equal to

ABSTRACT

Backgrounds

Hypertensive disorders of pregnancy, a major cause of maternal and neonatal morbidity worldwide are characterized by widespread maternal endothelial dysfunction and metabolic disorders (blood pressure and insulin resistance). Dysregulation in proteins (AKT and PI3K) involved in the insulin signaling pathway lead to insulin resistance, which is a common feature in the second half of most pregnancies complicated by preeclampsia and gestational hypertension. The objective of this study was to quantify microRNAs in serum and placental tissue of women with gestational hypertension (GH) and preeclampsia (PE).

Methods

This study is a prospective cross-sectional study involving 32 normotensive pregnant women (control), 32 women with preeclampsia (PE) and 28 with gestational hypertension (GH). The patients were recruited from a regional hospital in Durban, KwaZulu-Natal Province, South Africa. Serum and placental microRNA were quantified using RT-qPCR to compare levels of expression in the control, PE, and GH. In addition, a western blot analysis was carried out to investigate the levels of protein expression (AKT and PI3K) in the insulin signaling pathway.

Results

Serum, miRNA-222 quantitative real-time PCR expression levels were significantly lower in PE compared to normotensives ($p=0.0186$). miR-29 expression levels were significantly higher in PE ($p<0.0001$) and GH ($p<0.0001$) groups compared to normotensives. miR-181a serum expression levels of GH were significantly higher compared to normotensives ($p=0.0070$). Placental tissue expression showed significantly higher expression levels of miR-181a in PE ($p=0.0344$) and GH ($p=0.0344$) groups compared to normal controls. Western blot analysis of proteins showed a lower expression of AKT-serine and threonine in the PE ($p=0.0001$) *compared* to the normal control groups and significantly higher expression in the GH ($p=0.0001$) groups compared to the normal controls. Furthermore, the expression of the phosphatidyl-inositol-3 kinase (PI3K) was statistically lower in PE ($p=0.0001$) and GH ($p=0.0001$) compared to the normal controls.

Discussion/Conclusion

MicroRNAs may be used as potential biomarkers for PE and GH. The results of this study showed a correlation between the expression levels of miRNAs with AKT/PI3K in the insulin signaling pathway, reinforcing the existence of metabolic dysregulation in PE and GH.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Background

Hypertensive disorders of pregnancy (HDP) is a major cause of maternal and neonatal morbidity and mortality worldwide (Kintiraki *et al.*, 2015). It is described as an aberrant increase in blood pressure with proteinuric or non-proteinuric urine after the 20th week of gestation (Kintiraki *et al.*, 2015). The cause of HDP is still unknown and a major concern in both developed and underdeveloped countries, despite the extensive research to the study on these complications of gestation (Hutcheon *et al.*, 2011). The increased incidence of HDP in developing countries may be due to insufficient health care facilities, as well as poor antenatal care for women during the course of pregnancy (Ghulmiyyah and Sibai, 2012).

Hypertensive disorders of pregnancy includes conditions such as gestational hypertension, preeclampsia, chronic hypertension with superimposed preeclampsia, and eclampsia (Kintiraki *et al.*, 2015). In pregnancy, the placenta is a transitory organ that acts as a maternal/fetal barrier facilitating the exchange of gasses, nutrients, and wastes (Cross, 1998; Fu *et al.*, 2013). Therefore, appropriate placental development is essential for adequate fetal nourishment and proper growth. However, improper placental development can lead to preeclampsia (PE) (Steegers *et al.*, 2010; James *et al.*, 2010), intrauterine placental growth restriction (Zhong *et al.*, 2010; Mouillet *et al.*, 2010), and preterm birth (Lee *et al.*, 2011; Gauster *et al.*, 2012).

Preeclampsia is characterized by impaired placental spiral artery remodeling leading to decreased blood supply and oxygen needs of the placenta and the developing fetus. This diminished oxygen supply, in turn, results in placental hypoxia and ischemia (Naljayan and Karumanchi, 2013), releasing placental-derived particles and/or factors into maternal blood. These placental-derived particles contribute to the pathogenesis of PE as they have the capacity to initiate an exaggerated inflammatory response (Redman and Sargent, 2001), which leads to the widespread endothelial dysfunction that characterizes PE (Tannetta and Sargent, 2013). Also, implicated in the development of PE is an imbalance in the concentration of circulating pro-inflammatory molecules such as IL-8, IL-6, IL-1α, IL-12, TNF-α, and ILFN-γ.(Pinheiro *et al.*, 2013). Currently, there is no treatment for HDP, but these complications of pregnancy are known to resolve after delivery of the placenta (Magee *et al.*, 2014). It has also been reported that metabolic syndrome causes poor development of the placenta by targeting placental factors to initiate maternal inflammation and endothelial dysfunction (Jeyabalan *et al.*, 2015).

1.2 Prevalence

Globally, 5-8% of women are affected with hypertensive disorders in pregnancy (HDP) (Muti *et al.*, 2015). Most cases of maternal deaths that result from HDP, are recorded in low-income countries where the risk of a woman dying from complications of HDP is about 1 in 39 compared to 1 in 3800 as seen in developed countries (Abalos *et al.*, 2013). Furthermore, the Saving Mothers Report, (2015) has stated that 4452 maternal deaths occurred in South Africa from 2011-2013, and HDP were responsible for 14.8% of the deaths. In addition, 8.3% of maternal deaths within the same period in KwaZulu-Natal were caused by HDP (Saving-Mothers., 2015).

1.3 Classification of hypertensive disorders of pregnancy

Hypertensive disorders of pregnancy includes disorders such as gestational hypertension, chronic hypertension with superimposed preeclampsia, preeclampsia, chronic hypertension and eclampsia (Watanabe *et al.*, 2013).

1.3.1 Gestational hypertension

Gestational hypertension is hypertension superimposed after 20 weeks of gestation, with no traces of protein in the urine. Moreover, GH increases the risk of preeclampsia, with about 17% of women with GH acquiring preeclampsia (Hutcheon *et al.*, 2011; Saudan *et al.*, 1998). In some cases, there may be slight traces of protein in the urine of gestational hypertensive patients which predisposes them to progress to preeclampsia (Liu *et al.*, 2008).

1.3.2 Superimposed preeclampsia

Superimposed preeclampsia commonly occurs in women with chronic hypertension, resulting in maternal and fetal complications (Magee *et al.*, 2014). Abnormalities in the kidneys may manifest in superimposed preeclampsia before 20 weeks of gestation and is characterized by increased blood pressure and proteinuria following the 20^{th} weeks of pregnancy (Watanabe *et al.*, 2013).

1.3.3 Preeclampsia

Preeclampsia is a pregnancy disorder associated with hypertension (systolic blood pressure ≥140 mmHg and diastolic pressure ≥ 90 mmHg) and proteinuria (≥ 300 mg) arising at 20 weeks of gestation or more (North *et al.*, 2012). Women with PE are at a risk of developing severe complications such as high levels of hypertension, renal diseases, cardiac diseases, and diabetes mellitus (Barrett *et al.*, 2014). The etiopathology of PE is poorly understood but, it is speculated to involve poor trophoblast migration in the

placenta, and increased trophoblast cell apoptosis. This can lead to reduced uteroplacental perfusion pressure (RUPP) and placental hypoxia (Redman and Sargent, 2005; Whitley *et al.*, 2007).

1.3.3.1 Sub-classification of preeclampsia

Preeclampsia is sub-categorized according to onset and severity. Based on the onset of PE, it can be classified into:

i) Early onset preeclampsia - ≤ 33 weeks 6 days of pregnancy (Lisonkova and Joseph, 2013).
ii) Late onset preeclampsia- ≥ 34 weeks of pregnancy (Lisonkova and Joseph, 2013).

Based on the severity, this complication of pregnancy can be classified into mild PE and severe PE

i) Mild preeclampsia is characterized by a blood pressure > 140/90 < 160/110 mmHg, and proteinuria <300 mg but not more than 2.0 g in a 24-hour urine sample (++) on a dipstick) (Watanabe *et al.*, 2013).
ii) Severe preeclampsia is characterized by a blood pressure of greater than 160/110 mmHg and urinary protein concentration >2.0 g in a 24-hour urine sample (+++) on a dipstick) (Watanabe *et al.*, 2013).

1.3.4 Eclampsia

This is a condition characterized by seizures that occur before labor in hypertensive pregnancies (Watanabe *et al*., 2013). Factors such as cerebral edema and cerebral vasospasm are accountable for this condition (Sibai, 2003). Symptoms of eclampsia are more prominent in women during the antepartum period (Ghulmiyyah and Sibai, 2012).

1.4 Risk factors for hypertensive disorders in pregnancy

Various risk factors are defined as predisponent to hypertensive disorders in pregnant women globally. These factors include chronic hypertension, preeclampsia in previous pregnancies, multifetal gestation, obesity, nulliparity, and diabetes (Lykke *et al.*, 2009; Dalmáz *et al.*, 2011). Other factors that predispose women to preeclampsia are: Age (≥ 40 years), parity, multiple pregnancies, renal diseases (Duckitt and Harrington, 2005), insulin resistance, metabolic syndrome, genetic disposition, insufficient nutrient uptake such as calcium, antioxidant vitamins, and immune factors involved independently in the etiology of preeclampsia (Eskenazi *et al.*, 1991; López-Jaramillo *et al.*, 2005; Sánchez-Aranguren *et al.*, 2014).

1.5 Placental development and pathophysiology of hypertension in pregnancy

1.5.1 Trophoblast differentiation and placental development

In a normal pregnancy, development of the placenta begins with the occurrence of blastocyst implantation occurs (Norwitz, 2006). The blastocyst attaches to the decidua. This blastocyst is arranged around the interfaces between the cytotrophoblastic cells, the trophectoderm and the decidualized uterus (Clancy, 2009; Fu *et al.*, 2013; Red-Horse *et al.*, 2004). The trophectoderm provisionally differentiates as it invades the decidualized endometrium towards the inner third of the myometrium laterally, with maternal vasculature (Norwitz, 2006). Cytotrophoblast progenitor cells are located in the basal membrane of the placental villi, divide into two pathways; villous trophoblasts, and extravillous trophoblasts (Figure 1) (Knöfler, 2010). Fusion of the cytotrophoblast (CTBs) cells to the syncytiotrophoblast (STB) cells takes place in the villous pathway to configure a layer called the syncytial layer that subsequently acts as an outer layer of the placental villous tree. STB cells are responsible for the exchange of materials between the mother and developing embryo (Mayhew, 2014).

The CTBs change from a proliferative phase into a migration or invasion cells in the extravillous pathway (Kemp *et al.*, 2002; Fu *et al.*, 2013) Invasive cells are divided into the interstitial EVTs (iEVTs) and the endovascular EVTs (enEVTs). The iEVTs firmly fix the placenta to the uterus (Huppertz, 2007), while the enEVTs are responsible for the remodeling of the placental spiral arteries (Cartwright *et al.*, 2010).

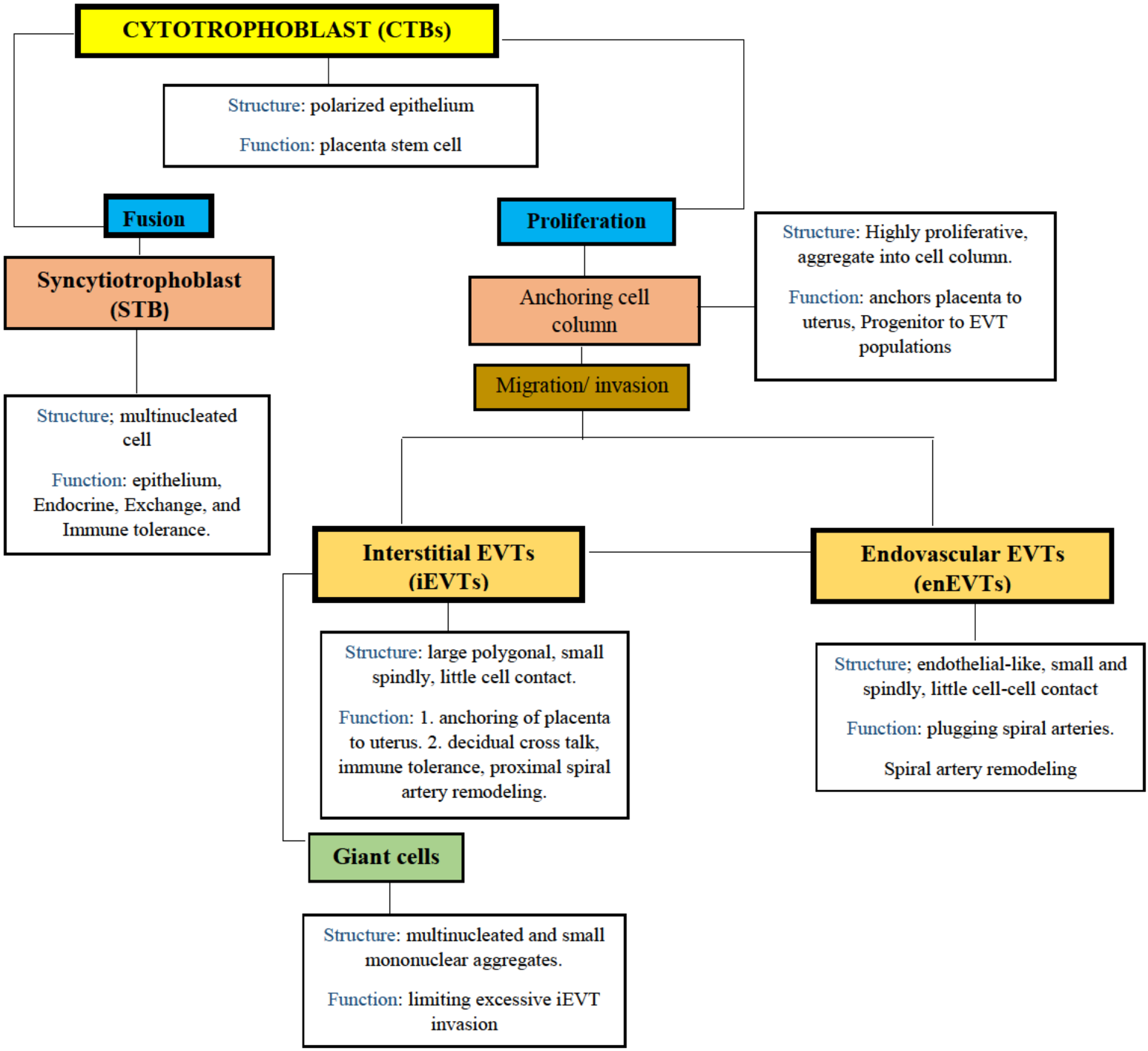

Figure 1: Schematic representation of Trophoblast differentiation. Adapted from (Fu *et al.*, 2013).The CBTs cells located at the base of the placenta villi and differentiate into two pathways: The villous and the extravillous trophoblasts. Fusion of the CTBs cells to the STB cells takes place in the villous pathway. The CTBs change from a proliferative phase into a migration or invasion cells in the extravillous pathway (Kemp *et al.*, 2002; Fu *et al.*, 2013) Invasive cells are divided into the interstitial EVTs (iEVTs) and the endovascular EVTs. iEVTs further divide into giant cells (enEVTs) (Huppertz, 2007).

1.5.2 Pathophysiology of HDP

The etiology of HDP is believed to be due to poor uteroplacental vascular remodeling, which causes reduced placental blood flow and consequently, hypoxia and ischemia (Redman and Sargent, 2010). Constricted uteroplacental flow is caused by insufficient invasion of the spiral arteries by the trophoblast cells resulting in ischemia (Barra *et al.*, 2012). Ischemia leads to placental cell damage, thus activating the discharge of material from the cell into the blood stream. This leads to activation of maternal vascular endothelium and causes endothelial dysfunction (Shah, 2007; Barra *et al.*, 2012).

Endothelial dysfunction can also be caused by oxidative stress. This occurs when pro-oxidants such as nitric oxide (NO), superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH), and peroxynitrite (ONOO−), are greater than antioxidants in the trophoblast cells(Myatt and Cui, 2004; Lappas *et al.*, 2010; Matsubara *et al.*, 2010; Kalyanaraman, 2013). Oxidative stress leads to activation of vascular inflammation. Endothelial dysfunction and inflammatory responses together are involved in the etiopathogenesis of PE (Sánchez-Aranguren *et al.*, 2014).

The pathology of preeclampsia involves two stages: The pre-clinical stage, and the clinical stage (Figure 2). The pre-clinical stage, which is referred to as stage one, occurs in early gestation and is characterized by poor placentation with decreased cytotrophoblastic cell invasion, irregular remodeling of spiral arteries as well as placental hypoperfusion (Tannetta and Sargent, 2013). Irregular remodeling of the spiral arteries result in oxidative stress (Redman, 2011), which subsequently leads to endothelial dysfunction.

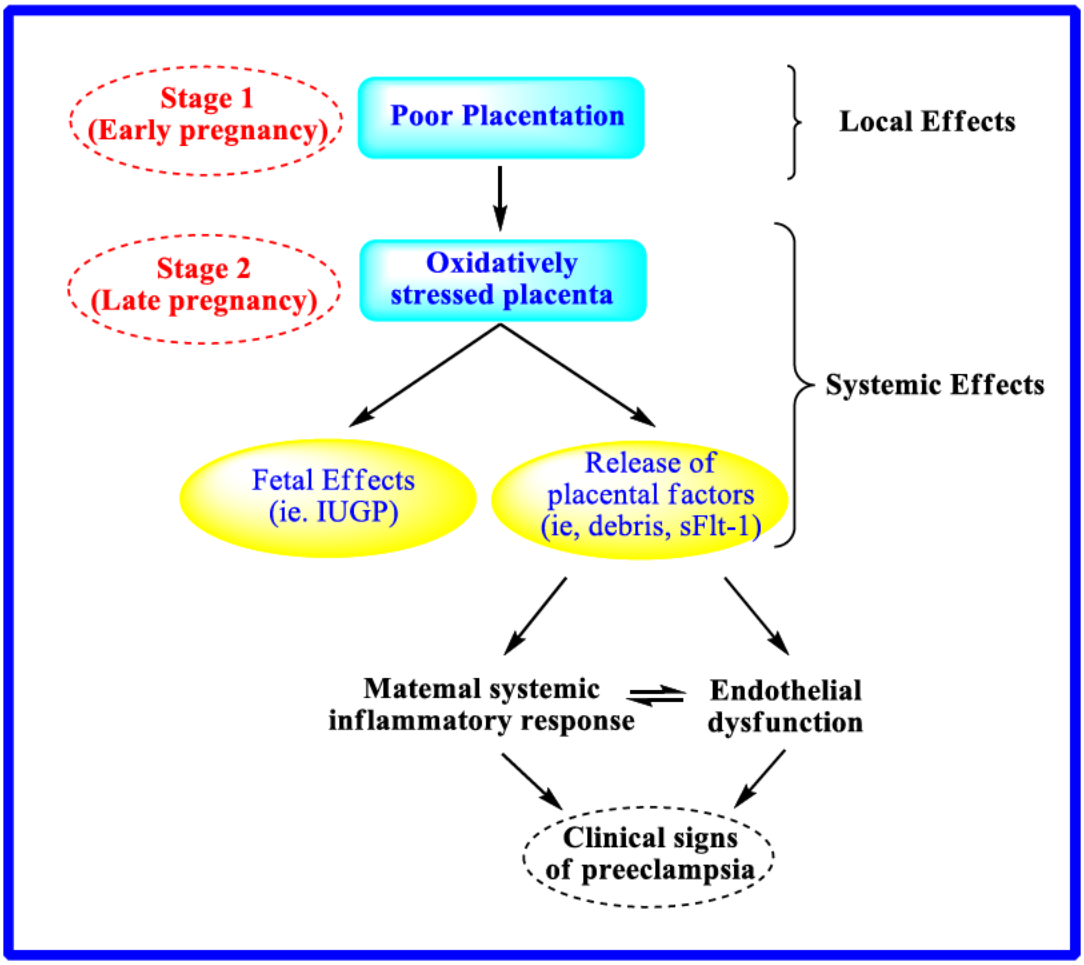

Figure 2. Diagrammatic representation of the stages of preeclampsia. Adapted from (Borzychowski *et al.*, 2006). Poor placentation results in abnormal remodeling and causes the release of placental particles or factors. These factors account for the initiation of systemic inflammatory response and dysfunction of maternal vascular endothelium which manifests as the clinical signs of in PE.

The clinical stage (second stage) takes place after endothelial dysfunction. In this stage, particles are released from the placenta into the maternal circulation (Granger *et al.*, 2001; Steegers *et al.*, 2010), leading to clinical manifestations. These particles include excess syncytiotrophoblast fragments (STBM), excess inflammatory cytokines, and activating immune cells (Roberts and Hubel, 2009). Other molecules released by the placenta are soluble endoglin (sEng), soluble vascular endothelial growth factor receptor-1 (sFlt-1). sFlt and sEng inhibit the release of the vascular endothelial growth factor (VEGF) thereby causing complications with the fenestrated endothelium and consequently protein leakage (proteinuria), (Craici *et al.*, 2013). While it is established that the angiogenesis is compromised in this disorder, the role of miRNAs in the pathophysiology of PE is of current scientific interest as a major contributor to various dysfunctional pathways.

1.6 MicroRNAs and their involvement in preeclampsia

MicroRNAs (miRNAs) are small non-coding RNAs with about 22 nucleotides, they perform posttranscriptional regulatory functions in normal physiological functions (Wahid *et al.*, 2010) and have a role in pregnancy disorders such as PE. Indeed, not only have miRNAs recently been reported as possible molecular biomarkers in the diagnosis of preeclampsia (Hu *et al.*, 2009), but a dysregulation in the expression levels have been observed in the placenta of patients with PE (Lim *et al.*, 2005; Hu *et al.*, 2009). About 157 miRNAs were identified in human placentas using real-time PCR (Pineles *et al.*, 2007). Differential expression of placental miRNAs was also reported in cases of severe PE (Wu *et al.*, 2012; Zhang *et al.*, 2015).

The process of angiogenesis and metabolic regulation are essential for placental development and a healthy pregnancy. It has also been stated that angiogenic factors play a role in the pathogenesis of PE. However, the factors leading to dysregulation of angiogenesis is poorly understood. It has been reported that miRNAs directly interact with angiogenic factors. These miRNAs include: miR-16, miR-26b, miR-29b, miR-181a, miR-195, miR-222 and miR-335 and are highly expressed in preeclamptic patients compared to normotensives. Genes associated with angiogenic factors like VEGF and PlGF are direct targets of these miRNAs. miR222, miR-195, and miR-335 directly affect cysteine-rich 61 (CYR61) protein, VEGF and PlGF. CYR61 protein plays crucial role in vascular integrity (Gellhaus *et al.*, 2006).

Patients with PE have been reported to have a reduction of invasive EVTs, inadequate remodeling of spiral arteries, and consequently, augmented apoptotic cells in the placenta (Norwitz, 2006). miRNAs may play a role in the impairment of physiological processes involved in pregnancy such as angiogenesis. Moreover, miRNA expression can be regulated by environmental factors (Donker *et al.*, 2007), epigenetic modifications (Seitz *et al.*, 2004; Fu *et al.*, 2013), as well as signaling pathways (Ji *et al.*, 2013). Figure 3 below indicates microRNA expression in the placenta and their involvement in PE pathogenesis.

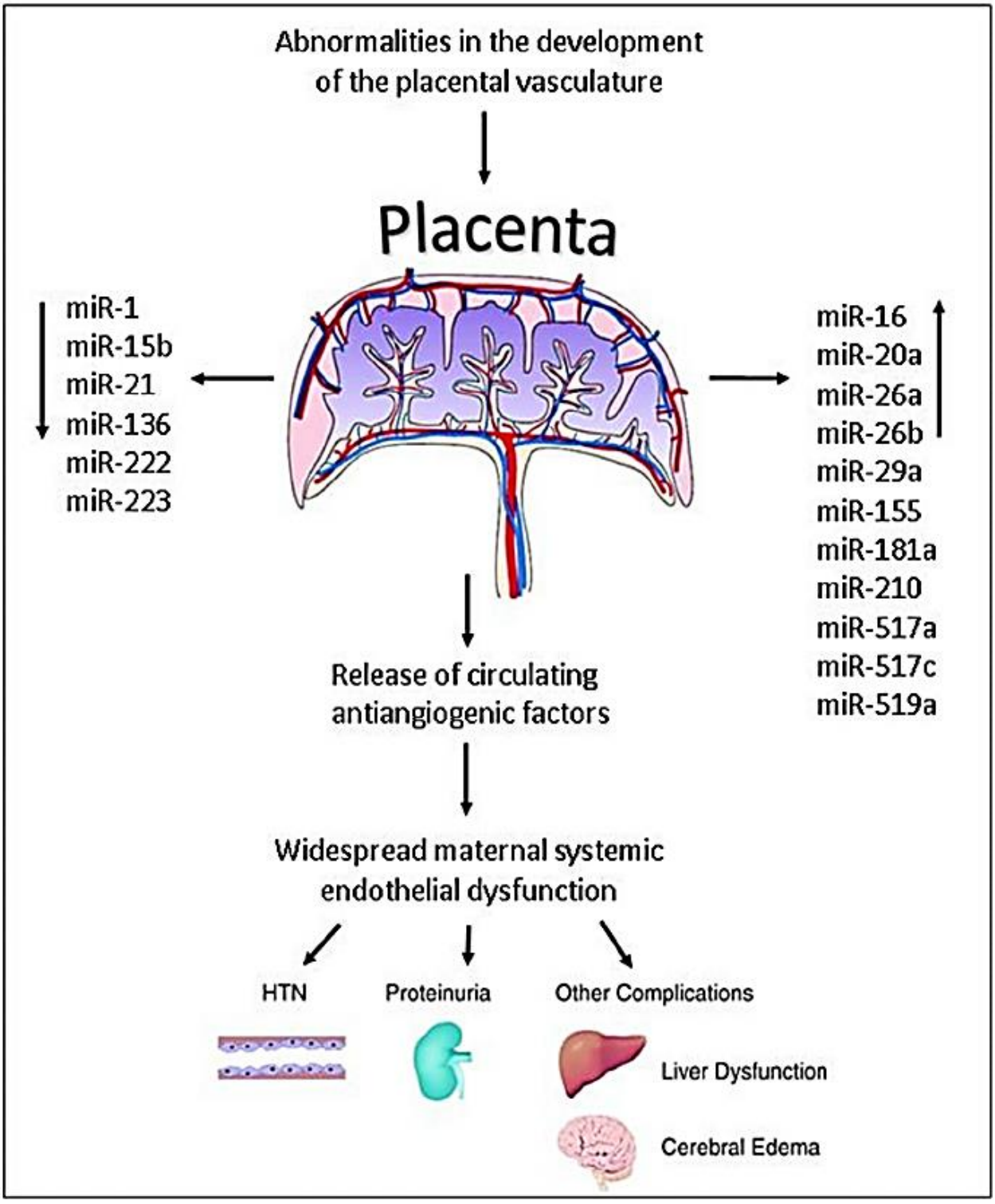

Figure 3: Schematic diagram of miRNAs involved in the Abnormalities of placental vasculature. Adapted from (Pillar *et al.*, 2015). Up or downregulation of these miRNAs cause the release of circulating antiangiogenic factors, which result in endothelial dysfunction in systemic blood vessels, such as the kidney, brain, and liver

miRNAs have been reported to affect metabolic pathways such as the insulin signaling pathway by targeting the proteins involved (Cudmore *et al.*, 2011). PE has been reported to be associated with metabolic syndrome and insulin resistance has also been reported in PE and in GH (Poornima and Rakesh, 2014). Furthermore, Hauth *et al.*, (2011) reported an increased risk of preeclampsia with increasing body mass index (BMI) in pregnant individuals. Glucose intolerance and hyperinsulinemia have been reported as being twice as high in HDP patients compared to normal pregnancy (Innes and Wimsatt, 1999; Kun, 2010). In addition, Kun, (2010), reported that insulin resistance was found to be elevated in GH patients compared to normotensives. Conversely, no insulin resistance was found in preeclamptic patients. It was further stated that insulin resistance may play a role in vascular dysfunction in gestational hypertensive patients by directly or indirectly altering physiological pathways related to sodium and water balance, as well as vascular resistance (Kun, 2010).

1.7 Insulin signalling pathway

Insulin signaling pathways involve a cascade of downstream signals (Chakraborty *et al.*, 2014). The proteins that are involved in the process are Insulin receptor 1(IRS1), Insulin receptor 1(IRS2), phosphatidylinositol-3 kinase (PI3K), Glucose transporter (GLUT-4) and AKT (Chakraborty *et al.*, 2014). The insulin signaling cascade involves several proteins that become activated when the insulin hormone binds to the insulin receptor, (α and β component). As insulin binds to the receptors, the component of the receptor that is in the cytoplasm becomes phosphorylated by tyrosine kinase (Jung and Choi, 2014). IRS protein then binds to the phosphorylated receptor and becomes phosphorylated as well by tyrosine kinase. An enzyme, PI3K binds to the phosphorylated IRS and becomes activated close to the plasma membrane where it binds to its substrate, phosphatidylinositol-4,5-biphosphate(PI(4,5)P2), forming a second messenger called PI(3,4,5)P3 (Jung and Choi, 2014).

The second messenger activates protein kinases and other enzymes. Protein kinase B, also known as AKT becomes activated and attaches to the membrane. It then becomes phosphorylated by a membrane located kinase (PDK1). AKT then activates the glycogen synthase by inactivating glycogen synthase kinase (GSK3) and glucose is converted to glycogen (glycogenesis).It also activates GLUT 4 translocation, thus promoting uptake of glucose by the cells. AKT also increases lipogenesis, decreases lipolysis and gluconeogenesis (Jung and Choi, 2014). Figure 4 below is a flow diagram illustrating the events occurring in the insulin signaling pathway.

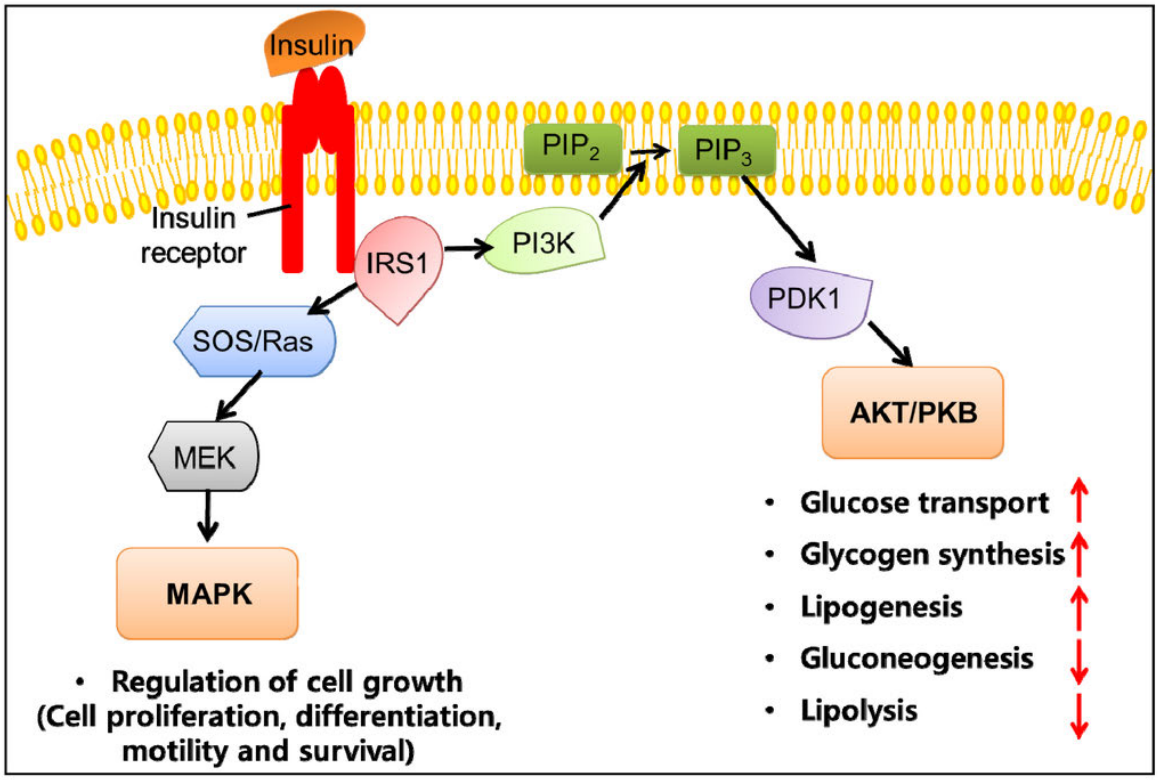

Figure 4: **Insulin Signaling Pathway.** Adapted from (Jung and Choi, 2014). AKT/PKB is the main glucose homeostasis regulator. The insulin hormone binds to the insulin receptor to activate a cascade of reactions that subsequently leads to the activation of AKT/PBK. AKT is essential for glucose transport activation, glycogenesis and lipogenesis. On the other hand, AKT deactivates gluconeogenesis and lipolysis for glucose regulation in the blood. Insulin resistance is defined as the inability of insulin to regulate blood sugar in the blood (Draznin, 2006). A decrease in AKT activity leads to increased amounts of glucose in the blood and this overpowers insulin function and results in insulin resistance.

1.8 miRNA function

Studies have identified miRNAs as key factors in the regulation of insulin pathways, insulin exocytosis, β cell functioning, and insulin synthesis by directly or indirectly targeting genes involved in these processes (Chen *et al.*, 2014). An upregulation or a downregulation of these miRNAs can lead to metabolic diseases. miRNAs perform posttranscriptional regulatory roles in normal physiological function by targeting messenger RNAs (mRNAs). Figure 5 below shows how miRNAs perform their function, followed by (Figure 6), which shows the miRNA maturation process.

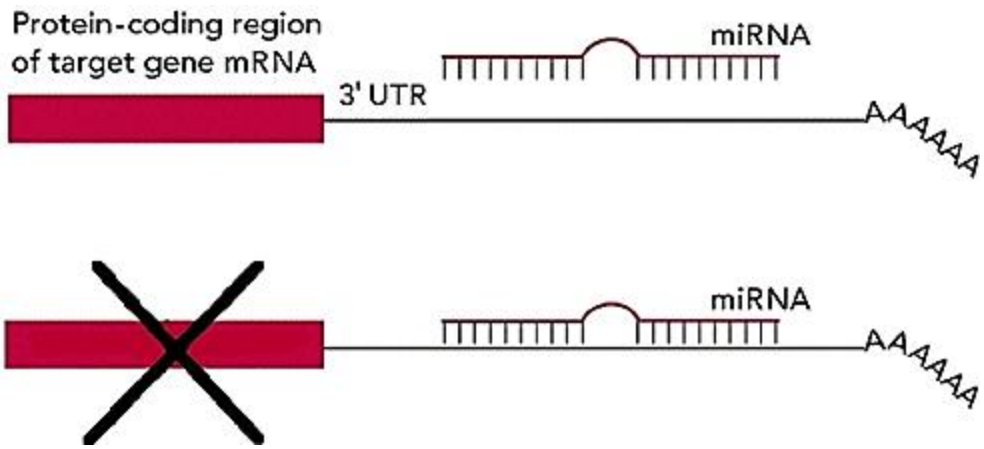

Figure 5: Schematic illustration miRNA function. Adapted from (Pillar *et al.*, 2015**)**. miRNAs bind to the three prime untranslated region (3'-UTR) of the mRNA, resulting in silencing or inactivation of the mRNA. This results in inhibition or repression of ribosomal translation into proteins.

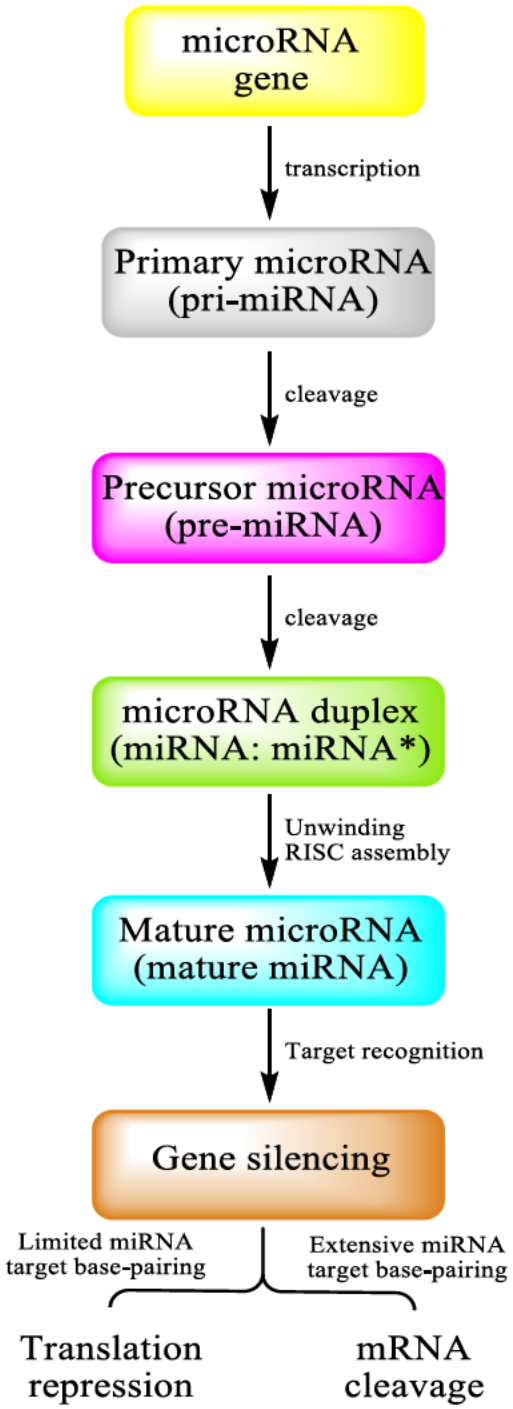

Figure 6: Diagrammatic presentation of miRNA maturation. Adapted from (MacFarlane and R Murphy, 2010). miRNA production begins in the nucleus, where primary miRNAs are cleaved into pre-miRNAs. Pre-miRNAs are processed into mature mRNAs and an effector RNA-induced silencing complex (RISC) is produced. Mature miRNAs direct the RISC complex to a target mRNA to silence it, inhibiting translation (MacFarlane and R Murphy, 2010).

The miRNA maturation process affects the expression of genes posttranslationally, giving rise to the degradation of mRNA. Single mRNA can be regulated by several miRNAs and similarly, a single miRNA can regulate several genes (Artmann *et al.*, 2012). Alteration in expression of specific miRNAs can lead to the development of certain diseases (Gilam *et al.*, 2013). It was reported that the expression levels of these mRNAs may be controlled by miRNAs at different levels in order to regulate signal transduction processes (Xu and Wong, 2008).

1.8.1 miR-222 as an antiangiogenic factor in preeclampsia

miR-222 was identified as an angiomiR in endothelial cells involved in angiogenesis in the placenta. miR-222 was found to have an inhibitory effect on the angiogenesis-stem cell factor (SCF), by downregulating the SCF receptor ligand. Placental development is dependent on a well-coordinated angiogenic process and if compromised, PE may develop (Fish *et al.*, 2008) Evidence has shown that an imbalance between angiogenic and antiangiogenic factors may disturb placental development and lead to hemolysis, vasoconstriction, increased permeability of the vasculature, and activation of the coagulation system. Since miR-222 inhibits angiogenesis, it is considered as an antiangiogenic factor (Rodríguez Santa *et al.*, 2015). It was reported that stimulation of 3T3-L1 adipocytes with increased concentrations of estrogen leads to a remarkable increase in miR-222 expression, leading to the conclusion that miR-222 may be related with estrogen-induced insulin resistance through its effects on ERα and GLUT 4 (Shi *et al.*, 2014).

1.9 Role of AKT/PI3K in preeclampsia

According to Cudmore *et al*., (2012), there is a loss of AKT activation in preeclamptic patients. Activation of AKT decreases soluble endoglin release (sEng), and an increased sEng increases the risk of preeclampsia. sEng is involved in vascular dysfunctions such as familial hypertension, atherosclerosis, systemic sclerosis, and malaria. (Fujimoto *et al.*, 2006; Dietmann *et al.*, 2009; Levine *et al.*, 2006). Pillar *et al*, (2015) studied the involvement of miR-29a, and miR-181a in PE and observed miR-29a and miR-181a were upregulated, miR-222 under expressed. These miRNAs were also shown to target the AKT/PI3K pathway.

1.9.1 miR-29a in the AKT/PI3K pathway

miR-29a was shown to have an effect on the insulin signaling pathway by targeting PI3K (Phosphatidylinositol 3-kinase) and AKT/PKB. miR-29a targets phosphoenolpyruvate carboxykinase 2 and the insulin-induced gene 1(INSIG1), in PE. INSIG1 plays an essential role in glucose homeostasis by upregulating insulin receptors, and the mediated regulation of phosphoenolpyruvate carboxykinase 2(PCK2), a key enzyme in gluconeogenesis and glycolysis (Krapivner *et al.*, 2007). An upregulation of miR-29a leads to reduced levels of the insulin-induced gene 1, affecting glucose homeostasis. Cells with a

downregulation of miR-29a showed higher levels of the INSIG 1(Zhao *et al.*, 2011). Other studies have indicated that miR-29a has an indirect inhibitory effect on AKT in the insulin signaling pathway, however, the mechanism of action is unknown (He *et al.*, 2007). miR-29a has an effect on PI3K by upregulating the p85α subunit (Park *et al.*, 2009). PI3K protein has two subunits: regulatory and catalytic subunits. The regulatory subunit is composed of p85α, p85β, p55α, p50α, and p55ϒ. The catalytic subunit is composed of p110α (Backer, 2010). An excess amount of regulatory subunit p85α leads to a decrease in AKT phosphorylation (Geering *et al.*, 2007).

1.9.2 miR-181a in the AKT/PI3K pathway and in preeclampsia

miR-181a targets B-cell CLL/lymphoma 2(BCL-2), T-cell leukemia/lymphoma 1A (TCL 1A) and it is upregulated in preeclampsia. miR-181a was reported to be highly expressed in placentas of patients with preeclampsia. It has also been observed that miR-181a increases the expression levels of IL-6 (Zhang *et al.*, 2015), leading to an imbalance in the concentration of the cytokine, thus contributing to PE pathophysiology (Liu *et al.*, 2012). Wang *et al*, (2011) found that the 'silent mating type information regulation 2 homolog-1' (Sirt-1) in hepatic cells of diabetic mice, decreases when miR181a is upregulated, disabling glucose metabolism and the insulin signaling pathway. Deficiency of Sirt 1 results in disruption of AKT signaling and this leads to hyperglycaemia and insulin resistance (Zhou *et al.*, 2012).

1.10 Applications of miRNAs

Recently, miRNAs have been discovered as biomarkers for various diseases. miRNAs show increased stability in paraffin-embedded tissues from clinical and human samples (Mitchell *et al.*, 2008) enhancing the fact that miRNAs can be used as diagnostic tools for diseases. Moreover, miRNAs can also be used as therapeutic strategies for certain diseases. miRNAs have been classified as RNAi-based therapeutics whereby miRNAs are manipulated to target and regulate hundreds of transcripts to cease dysregulated pathways (Hydbring and Badalian-Very, 2013).

miRNAs are highly stable in circulation and are therefore capable of regulating and dysregulating different pathways. The purpose of this study is to investigate the expression levels of miR-29a, miR-181a, and miR-222 in preeclamptic and gestational hypertensive patients and to correlate them with the key metabolic proteins (AKT/PI3K) in the insulin signaling pathway.

1.11 Hypothesis of the study

Dysregulation of miRNA expression is involved in the pathogenesis of preeclampsia and gestational hypertension.

1.12 Aim of study

An investigation of the expression levels of miR-29a, miR-181a, and miR-222 as well as the expression of AKT and PI3K in preeclamptic and gestational hypertensive patients.

1.13 Objectives

I) To determine the serum expression levels of miR-29, miR-181a, and miR-222 in preeclamptic patients and gestational hypertensives using real-time PCR.

ii) To quantify the placental expression levels of miR-29a, miR-181a, and miR-222 in preeclamptic and gestational hypertensive patients using real-time PCR.

iii) To determine the placental tissue expression levels of AKT and PI3K subunit of patients with preeclampsia and gestational hypertension, using western blot analysis.

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2.1 Differential expression of microRNAs associated with the insulin signaling pathway in preeclampsia and gestational hypertension

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Abstract

Preeclampsia (PE) is the most common direct cause of maternal morbidity and mortality. Insulin resistance characterizes the second half of most pregnancies, including PE and GH. The role of microRNAs (miRNAs) in insulin resistance associated with these disorders is unclear. Therefore, the aim of this study was to determine miRNAs expression levels in placental tissue and serum samples from normotensive (n = 32), PE (n = 32) and GH (n = 28) in South African women of Zulu ethnicity. miRNAs were isolated from these samples using the mirVana kit, and qPCR was conducted to determine their expression. Serum, miRNA-222 quantitative real-time PCR expression levels were significantly lower in PE compared to normotensives (*p=0.0186*). MiR-29 expression levels were significantly higher in PE *(p<0.0001)* and GH *(p<0.0001)* groups compared to normotensives. MiR-181a serum expression levels of GH were significantly higher compared to normotensives (*p=0.0070*). Western blot analysis of placental tissue showed up or down regulation of proteins in PE vs normotensives and GH vs normotensives. Results in placental tissue showed significantly higher expression levels of miR-181a in PE *(p=0.0344)* and GH *(p=0.0344)* groups compared to normal controls. We found a lower expression of AKT-serine and threonine in the PE *(p=0.0001)* compared to the normal control groups and significantly higher expression in the GH groups compared to the normal controls (*p=0.0001)*. The expression of the phosphatidyl-inositol-3 kinase (PI3K) was statistically lower in PE *(p=0.0001)* and GH *(p=0.0001)* compared to the normal controls. Differential expression of miRNAs in correlation with the insulin signaling pathway was found, reinforcing the existence metabolic dysregulation in PE and GH. The present study, for the first time, shows the differential expression of circulating miRNAs in GH. Therefore, these miRNAs may be used as potential biomarkers for PE and GH.

Keywords; MiR-222, Preeclampsia, MiR-181a, Gestational hypertension, PI3K

Introduction

Hypertensive disorders of pregnancy (HDP) affect women worldwide (Beltran *et al.*, 2013) and includes conditions such as preeclampsia (PE), gestational hypertension (GH), chronic hypertension with superimposed PE, the HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets), white coat hypertension and eclampsia (Tranquilli *et al.*, 2014). Globally PE affects about 8-10% of all pregnancies. In Africa, about 270,000 maternal deaths are due to PE (Vata *et al.*, 2015). HDP are also the commonest direct cause of maternal deaths in South Africa,

accounting for approximately 14% of all maternal deaths (Deaths, 2015). Furthermore, babies delivered by women who had PE, have a risk of developing cardiovascular disorders (Gathiram and Moodley, 2016). Currently, there is no treatment for PE, with the delivery of the fetus and placenta being the only known cure (Magee *et al.*, 2014).

The exact etiology of PE is not known, and therefore, more research is needed. The pathology of PE is categorized into two stages: stage one occurs in early gestation and is characterized by poor placentation with decreased cytotrophoblastic cell invasion, which results in inadequate remodeling of placental spiral arteries, and hypoperfusion of the placenta (Tannetta and Sargent, 2013). Altered spiral artery remodeling results in diminished blood flow to the placenta, hypoxia of trophoblast cells and increased placental oxidative stress (Redman, 2011). The second stage of PE is characterized by increased oxidative stress which leads to the destruction of placental cells, followed by the release of cellular debris into maternal circulation (Tannetta and Sargent, 2013). This debris such as syncytiotrophoblast microparticles and cell-free nucleic acid have the potential to stimulate inflammatory response leading to endothelial dysfunction (Tannetta and Sargent, 2013). Studies have reported the involvement of microRNAs in the pathophysiology of PE (Lim *et al.*, 2005; Hu *et al.*, 2009; Vashukova *et al.*, 2016).

miRNAs are non-coding RNAs that function posttranscriptional regulators in biological processes by degradation and translational inhibition of mRNAs (Wahid *et al.*, 2010). It has been reported that miRNAs are expressed at different levels in the placenta for each type of pregnancy-related pathology (i.e. PE) (Wu *et al.*, 2012). Approximately, 157 miRNAs have been identified in the placenta using real-time polymerase chain reaction (PCR) (Pineles *et al.*, 2007). They are implicated in placental development through cellular differentiation, proliferation, apoptosis, invasion and angiogenesis (Fu *et al.*, 2013). Furthermore, dysregulation of miRNA expression was identified in preeclamptic placentae (Zhang *et al.*, 2015). Angiogenic factors such as vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) are essential for placental development during pregnancy, however, these factors are downregulated in PE (Cartwright *et al.*, 2010). It has been reported that genes interrelated with angiogenic factors are direct targets of these miRNAs (Gellhaus *et al.*, 2006). The microRNAs associated with angiogenic factors include miR-16, miR-26b, miR-29b, miR-181a, miR-195, miR-222 and miR-335.

miRNAs have also been shown to target metabolic pathways such as the insulin signaling pathways (AKT/PI3K pathway) in PE (Cudmore *et al.*, 2011) and in gestational diabetes (GDM) (Pillar *et al.*, 2015).These include miR-29a which has been reported to target the AKT/PI3K protein when upregulated, resulting in a decrease in the protein's expression (He *et al.*, 2007; Backer, 2010; Geering *et al.*, 2007). Loss of AKT activation has been

found in PE patients. Inactivation of AKT increases the levels of anti-angiogenic factors such as soluble endoglin release (sEng), which in turn, increases the risk of PE (Dietmann *et al.*, 2009). While there is some literature on miRNAs implicated in the metabolic dysregulation and angiogenesis in PE, there is little on GH, which may progress to PE. Importantly, a 'comorbidity" may exist between the two states, and microRNAs may serve as potential biomarkers for these two conditions. Hence in the present study, miRNA expression levels in placental tissue and serum samples from women with GH and PE patients were investigated using quantitative real-time PCR. Secondly, the effects of placental microRNAs on the proteins associated with the insulin signaling pathway were also investigated using western blot analysis.

Study Design, Practical Aspect

Regulatory Permissions

Ethical approval was received from the University of KwaZulu-Natal Biomedical Research Ethics Committee (BE229/16), South Africa. Health authority permission was also obtained and all research participants provided written informed consent.

Study Population

This is a cross sectional study and participants were recruited at a regional hospital in Durban South Africa. Participants included normotensives with no obstetrical or medical complications (n=32), GH (n = 28), and PE (n= 32). All participants were aged between 17-45 years. Patients with chronic hypertension, previous history of PE, diabetes and other cardiovascular disorders were excluded. GH was defined as a blood pressure of ≥ 140/90mmHg with no proteinuria (Tranquilli *et al.*, 2014). Preeclampsia was defined as a sustained blood pressure of ≥ 140/90mmHg with proteinuria of at least 300 mg after 20 weeks of pregnancy (Tranquilli *et al.*, 2014). Preeclampsia was further subdivided into mild and severe PE. Mild PE was defined by a blood pressure of ≥ 140/90 mmHg but ≤ 159/109 mmHg, and proteinuria of 300mg or more but not exceeding 2.0g in a 24-hour urine sample (Tranquilli *et al.*, 2014). Severe PE was defined by a blood pressure ≥ 160/110 mmHg and urine protein concentration of > 2.0g in a 24-hour urine sample (Tranquilli *et al.*, 2014).

Sample collection

Blood samples were collected in 6ml serum tubes from the research participants. Processing for serum extraction was done at 2500 rpm for 10 min at room temperature, an hour after collection. The extracted serum was stored immediately at -80◦C. Placentae were also collected from patients who had elective cesarean deliveries. Small

pieces of tissue from the central part of the placenta were extracted within 15 minutes of delivery, washed with Phosphate-buffered saline (PBS) and immediately stored in liquid nitrogen for western blot analysis. Pieces of the same tissue were also washed with PBS as well and stored in RNAlater for microRNA extraction.

MicroRNA isolation

MicroRNAs were isolated from 10mg of placental tissue and 100µl serum using the mirVana Kit according to the manufacturer's instructions (ThermoFisher Scientific) and was eluted in 50µl of nuclease free water. A synthetic spike-in control miRNA *(Caenorhabditis elegans*-miR-39*)* was added for normalization following a published protocol (Kroh *et al.*, 2010). Because of the likelihood of the presence of inhibitors during RT-PCR, a synthetic spike-in miRNA was used as an exogenous control to prevent loss of miRNA during the isolation process (Tomasetti *et al.*, 2012).

Quantitative Real-Time Polymerase Chain Reaction Analysis for miRNAs

Total miRNAs from each sample was reverse transcribed into cDNA using iScript cDNA Synthesis Kit according to manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA). Quantification of miRNAs was done using SYBER-Green 1 master mix with the light cycler 96 (Roche). 2µl of cDNA was used for qRT-PCR. The master mix contained 10µl SYBER- Green, 2µl forward respectively and 4µl RNase-free water for a final volume of 20µl. All samples were run in duplicates. The 3 step amplification procedure was performed at 95° C for 15 min, 60° C for 30 sec, and 72° C for 30 sec. The U6 small nuclear RNA (RNU6) and c-miR-39 were used to determine relative miRNA expression following a protocol by Kroh *et al*., (2010). The miRNAs of interest were as follows: miR-222; Forward (TCCAGTGCAGGGTCCGAGGTAT); Reverse primer (TAATAGAAAGCTACATCTGGCTACTGGGT); miR-29a: Forward (GTAGCACCATCTGAAATCGGTT) Reverse primer (GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAACCG); miR-181a: Forward (ACACTCCAGCTGGGAACATTCAACGCTGTCG); Reverse primer (CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACTCACCG).

Western Blot Analysis

Placental tissue was lysed using 2.5 ml RIPA buffer (Sigma), and 250 µl protease and phosphatase inhibitor (Abcam). The tissue was homogenized on ice and centrifuged at 12000 rpm at 4° C for 10 minutes. The supernatant was extracted and the pellet was discarded from the solution. Protein quantification was then carried out using the RC DC Assay method (Bio-Rad Laboratories, Hercules, CA, USA). Protein standard 1 was used with a concentration of

1.47 mg/ml (Bio-Rad Laboratories, Hercules, CA, USA). Proteins (15µg) were loaded onto precast gels (Any kD, Bio-Rad) and separated using sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) electrophoresis. The proteins were transferred using polyvinylidene fluoride (PVDF) membranes. The chemiluminescent blocking agent (Bio-Rad Laboratories, Hercules, CA, USA) was added onto the membranes and placed onto a shaker for 1 hour at room temperature. Membranes were treated with primary antibodies (Anti-AKT1 (phospho S473)- Rabbit monoclonal, Anti-AKT1 (phospho T308) - Mouse monoclonal, Anti-PI 3 Kinase p85 alpha- Rabbit monoclonal) (Cell Signaling Technology) overnight at 4° C, followed by an incubation of 1 hour at room temperature with a secondary antibody (Goat Anti-Goat IgG H&L (HRP) (Cell Signaling Technology). Lastly, membranes were incubated in ECL substrate and viewed on the Chemidoc Touch Imager system (Bio-Rad Laboratories, Hercules, CA, USA). Band intensity was measured with an image J version 6 analyzer.

Statistical Analysis

Results were expressed as means ±SEM. The statistical significance of the difference in expression levels of miRNAs in all samples groups was performed using the t-test (non-parametric tests), using Graph pad prism 5 software (Graph Pad software, San Diego, CA, USA). A p-value < 0.05 was considered as statistically significant.

Results

Table 1 shows patient demographics and their clinical characteristics. There was no statistical difference in maternal and gestational age among the study groups. There were also no statistical differences in the BMI among the patient groups. However, there were statistical difference in the systolic blood pressure and the diastolic blood pressure in the PE and GH groups compared to normotensives, and in the GH group compared to the PE group ($p<0.05$)

Figure 1 indicates the expression levels of miR-222 in the serum of normotensive, GH, and PE groups. There was a significant decrease in the expression levels of miR-222 in the PE group compared to normotensive group ($p<0.05$). There was no statistical significance between the expression levels of the GH and normotensive groups. Similarly, no significant difference between GH and PE patients was observed.

Serum miR-29a expression levels for the normotensives, GH and PE groups are shown in Figure 2. There is a significant increase in the expression levels of the PE group compared to the normotensives ($p<0.0001$). There was also a significant increase in the expression levels of miR-29a in the GH groups difference compared to the normotensives ($p<0.0001$). The miR-29a expression levels of the GH group did not statistically differ from that of the PE group ($p=0.7547$).

Figure 3 shows the serum expression levels of miR-181a in normal, GH and PE groups. There was a significant increase in the expression levels of the GH group compared to the normotensives group ($p=0.0070$). There was no significant difference between the normotensives group and the PE group ($p=0.2164$). A statistical difference between the PE group and the GH group was also observed, with an increase in the expression levels of the GH compared to the PE group ($p=0.0006$).

The placental miR-181a expression levels are shown in Figure 4 for the normotensive, GH and PE groups. There was a statistical difference between the normotensive and PE groups ($p=0.0344$), and there was a significant difference between the normotensive group and the GH group ($p=0.0344$). There was no significant difference between the GH group and the PE group.

Figure 4 (a, b, c, d) shows the western blot analysis of the expression levels of phosphorylation in AKT-serine, AKT-Threonine, and PI3K in the placenta of the normotensive, GH and PE groups. Figure 4 (b) shows the expression levels of phosphorylation of AKT-serine was downregulated in PE compared to normotensive. However, the expression levels of phosphorylation of AKT-serine was upregulated in GH compared to normotensives ($p=0.0001$). Figure 4 (c) shows the expression levels of phosphorylation of AKT-Threonine was downregulated in PE compared to normotensive. However, the expression levels of phosphorylation of AKT-Threonine was upregulated in GH compared to normotensives ($p=0.0001$). Figure 4 (d) indicates the expression of PI3K in the placenta of normotensives, GH and PE groups. The expression levels of phosphorylation of PI3K was downregulated in PE and GH compared to normotensive ($p=0.0001$). A significant decrease was also observed when the GH group was compared to the PE group ($p=0.0010$).

Table 2 shows the correlation between the miRNAs investigated and the key proteins (AKT and PI3K) in the insulin signaling pathway. A significantly strong negative correlation was observed between miR-222 levels and the AKT/PI3K proteins in normotensives ($r= -1.0$, $p <0.01$). MiR-222 levels in the GH group showed a significantly strong positive correlation with the AKT/PI3K proteins ($r=1.0$, $p<0.01$). A significantly strong negative correlation was found between miR-222 levels in the PE group and the AKT/PI3K proteins ($r= -1.0$, $p<0.01$). miR-29a levels in the normotensives showed a significantly strong positive correlation with AKT/PI3K proteins ($r= 1.0$, $p<0.01$). In the GH group, there was a significantly strong negative correlation between the levels of miR-29a and AKT/PI3K proteins ($r=-1.0$, $p<0.01$). A significantly strong negative correlation was observed in the level of miR-29a and AKT/PI3K proteins in the PE group ($r=-1.0$, $p<0.01$). The level of miR-181a in the normotensives and the AKT/PI3K proteins

showed a significantly strong positive correlation ($r=1.0$, $p<0.01$). There was a significantly strong negative correlation in the levels of miR-181a and AKT/PI3K in the GH as well as the PE group ($r= -1.0$, $p<0.01$).

Discussion

Preeclampsia (PE) and GH are hypertensive disorders of pregnancy characterized by widespread maternal endothelial dysfunction and metabolic disorders (blood pressure and insulin resistance) (Poornima and Rakesh, 2014; Murphy *et al.*, 2015). miRNAs are emerging as critical regulators of biological functions, alteration in miRNA expression may contribute to the pathogenesis of PE (Pineles *et al.*, 2007; Gunel *et al.*, 2011; Pillar *et al.*, 2015; Munaut *et al.*, 2016). The three microRNAs in the present study were selected based on previous studies, because of their involvement in angiogenesis and insulin resistance (Zhu *et al.*, 2009; Pillar *et al.*, 2015; Yang *et al.*, 2015; El-Shorafa and Sharif, 2016) and the results were validated using RT-qPCR. However, miRNA expressions in the placenta were investigated previously on gestational diabetes and PE (Pillar *et al.*, 2015; Chen *et al.*, 2014; Sheikh *et al.*, 2016), but not in GH. Importantly, gestational hypertensive patients are reported to develop PE in their third trimester or in their next pregnancy (Mudjari and Samsu, 2015). In the present study, we investigated the expression levels of miR-222, miR-29a, and miR-181a in PE and GH because of the assertion that these two conditions are associated with endothelial dysfunction as well as metabolic disorders caused by these miRNAs (Yelumalai *et al.*, 2010; Pillar *et al.*, 2015). A focus on the following pathogenic domains viz. hypoxia, angiogenesis, and immunology have been highlighted.

The findings of the present study show that miR-222 was significantly downregulated in the serum of preeclamptic patients compared to normotensive patients ($p =0.0186$). There were no significant differences in the serum expression levels of miR-222 between the normotensive and the GH groups ($p=0.3260$). Notably, the role of miR-222 appears to be important in pregnancy success (Sang *et al.*, 2013). Upregulation of miR-222 has been reported to increase estradiol secretion, while downregulation of miR-222 decreases estradiol secretion. It has been suggested that an upregulation of estradiol secretion is important for the early stages of development in pregnancy (Sang *et al.*, 2013). In PE, the placenta is underdeveloped, this might be due to the downregulation of miR-222. It has also been reported that miR-222 plays a role in endometrium stem cell differentiation, which makes it essential for decidualization and implantation (Qian *et al.*, 2009). Our results are in keeping with previous reports (Pillar *et al.*, 2015; El-Shorafa and Sharif, 2016). Furthermore, this specific miRNA has been identified as an angiomiR, and it

regulates angiogenesis by inhibiting the angiogenesis-dependent Stem Cell Factor (SCF) through targeting c-KIT, which is an SCF receptor ligand (He *et al.*, 2007). Our findings also showed that miR-222 was downregulated in serum samples of the PE group and this might play a role the inhibition of angiogenesis.

In the current study, miR-29a was upregulated in the serum of preeclamptic and GH patients compared to the normotensives. Increased miRNA-29a expression has been reported to result in an upregulation of PI3K and downregulation of AKT target genes in the insulin signaling pathway (Geering *et al.*, 2007). This disrupts the insulin signaling pathway and contributes to the pathogenesis of PE. MiR-29a has been reported to directly target the regulatory subunit found in PI3K known as p85α (Geering *et al.*, 2007). According to Geering *et al*., (2007), upregulated miR-29a directly increases p85α expression, resulting in downregulation of the AKT protein. The physiological function of AKT is to regulate cellular metabolism through the insulin signaling pathway (Boucher *et al.*, 2014). In the present study, the western blot results showed that AKT is under-expressed in the placental tissue of preeclamptic patients compared to normotensives. These results are in keeping with that of Geering *et al*., (2007), Cudmore *et al.,* (2011) and Pillar *et al*., (2015). It has been stated that loss of AKT activity increases the levels of soluble endoglin release (sEng), which in turn, increases the risk of PE (Dietmann *et al.*, 2009). Soluble endoglin release inhibits angiogenic factors like the vascular endothelial growth factor (VEGF) (Rădulescu *et al.*, 2016). Vascular endothelial growth factor has been reported to induce a variety of proteins including PI3K and also regulate endothelial cell proliferation and cell differentiation via the PI3K/AKT signal transduction pathway (Gerber *et al.*, 1998).

Inhibitors of PI3K (wortmannin or LY 294002) lead to increased cellular apoptosis, and this indicates that VEGF cannot function in the absence of PI3K.VEGF has also been reported to decrease sEng release in endothelial cells (Gerber *et al.*, 1998; Cartwright *et al.*, 2010) Endothelial cell apoptosis is diminished in the presence of VEGF and PI3K, PI3K is responsible for AKT activation (Gerber *et al.*, 1998). Reports have shown that AKT activation results in a decrease in soluble endoglin release in endothelial cells (Cudmore *et al.*, 2011). In our findings, miR-29a was upregulated in both GH and PE serum samples, this might be associated with vascular dysfunction in PE and GH.

In the present study, serum expression levels of miR-181a in GH were upregulated compared to the levels in PE and normotensive groups, there was no significant difference in the levels of miR-181a in the serum of pre-eclamptic patients and normotensives. However, miR-181a was upregulated in placentas of preeclamptic and GH groups compared to the normotensive ($p=0.0070$). The placental findings in the present study are consistent with the

results obtained by others (Enquobahrie *et al.*, 2011; Ishibashi *et al.*, 2012; Wang *et al.*, 2012; Luo *et al.*, 2014; Xu *et al.*, 2014; Pillar *et al.*, 2015). According to Liu *et al*., (2012), upregulation of miR-181a in PE increases the levels of IL-6, which is involved the with the ATA-AA gene responsible for the release of anti-angiogenic factors such as sFlt-1 and sEng by activating the AT1 receptor. These factors have been reported to inhibit angiogenesis in PE (Cartwright *et al.*, 2010). Similar to miR-29a, miR-181a has been reported to be involved in glucose homeostasis in the insulin signaling pathway (Wang *et al.*, 2011). An upregulation of miR-181a has been reported to decrease the expression of the silent mating type information regulation 2 homologs (Sirt-1) and this was found in diabetic mice (Wang *et al.*, 2011). A deficiency of Sirt-1 has been reported to disable AKT signaling (Zhou *et al.*, 2012). In this study, a significant decrease in AKT expression was noted in the placenta of the PE group, compared to normotensives (p=0.0001). In contrast, AKT expression levels were significantly higher in GH compared to the levels in normotensives and PE groups (p=0.0001). This finding may be an indication that the metabolic pathways involved in the pathogenesis of the two hypertensive disorders in this study are different.

In the current study, a correlation was done to investigate the relationship between the miRNAs and the key proteins in the insulin signaling pathway (AKT and PI3K). A significantly strong negative correlation was observed between the levels of miR-222 in the normotensive and PE groups, and the AKT/PI3K proteins ($r = -1.0, p < 0.01$). This indicates that the miR-222 in the normotensive and the PE groups are inversely proportional to the AKT/PI3K proteins. A decrease in miR-222 results in an increase in the AKT and PI3K proteins. MiR-222 in the GH group has a significantly strong positive correlation with the AKT/PI3K pathway ($r = 1.0, p<0.01$). These results indicate that miR-222 in the GH group is directly proportional to the AKT and PI3K proteins. Therefore, a decrease in miR-222 may lead to a decrease in AKT.

A significantly strong positive correlation was found between the miR-29a in normotensives and the AKT/PI3K pathway ($r =1.0, p<0.01$), but a significantly strong negative correlation was found in miR-29a (GH and PE) and AKT/PI3K ($r = -1.0, p<0.01$). miR-181a in normotensives was found to have a significantly strong positive correlation with the AKT/PI3K pathway ($r =1.0, p<0.01$). However, miR-181a in GH and PE had a significantly strong negative correlation with the AKT/PI3K pathway ($r= -1.0, p<0.01$). The significantly strong negative correlation observed between the levels of miRNAs in PE and GH investigated in this study, and the key proteins (AKT and PI3K) in the insulin signaling pathway show that the dysregulation of these miRNAs disrupts the insulin signaling pathway, thus contributing to the pathogenesis of this pregnancy disorder. The strength of the present study

is that as far as we are aware, this is the first report on investigations into miRNAs in women with gestational hypertension.

Conclusion

miRNAs play an important role in the pathophysiology of GH and PE, in that miRNA expression is dysregulated in these conditions. miRNAs may be useful as potential biomarkers for the detection of hypertensive disorders of pregnancy.

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Figures and table legend

Figure 1 serum expression levels of miR-222 in normal, gestational hypertensive (GH), and preeclamptic (PE) patients.

*normal vs preeclamptic (p< 0.05).

Figure 2 serum expression levels of miR-29a in normal, gestational hypertensive (GH), and preeclamptic (PE) patients.

***normal vs GH, and, *** normal vs PE with a (p < 0.05).

*shows significance of p<0.05 between groups; *** shows a significance of p<0.01.

Figure 3 Serum expression levels of miR-181a in normal, gestational hypertensive (GH), and preeclamptic (PE) patients.

**normal vs GH (p < 0.05).

Figure 4 Placental expression levels of miR-181a in normal, gestational hypertensive (GH), and preeclamptic (PE) patients.

**normal vs preeclamptic and GH (p< 0.05).

Figure 5 (a) represented the immunoblots for placental AKT-Serine, AKT-Threonine and PI3K of normal, gestational hypertensive (GH), and preeclamptic (PE) patients (fold change relative to □-Actin). Figure 5 (b) Phosphorylation of placental AKT-Serine in normal, gestational hypertensive (GH), and preeclamptic (PE) patients. *Normal vs GH (P<0.05), *normal vs PE (P<0.05). **Figure 5(c) Phosphorylation of placental AKT Threonine in normal, gestational hypertensive (GH), and preeclamptic (PE) patients**. *Normal vs GH (P<0.05), *normal vs PE (p<0.05). **Figure 5(d) Phosphorylation of placental PI3K in normal, gestational hypertensive (GH), and preeclamptic (PE) patients**. *Normal vs GH (P<0.05), *normal vs PE (P<0.05).

Table 1 Patient demographics and clinical characteristics

*Control vs GH (p<0.05); #control vs PE (p <0.05); µ GH vs PE (p <0.05)

BMI; body mass index, Values are expressed as mean ± standard deviation, and number of occurrence.

Table 2 Correlation between placenta miRNAs levels and protein in the (A) Normotensives, (B) GH, and (C) PE Groups

Pearson correlation (r); **Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed). Abbreviations; GH, gestational hypertension, PE, Pre-eclampsia, n, number of patients.

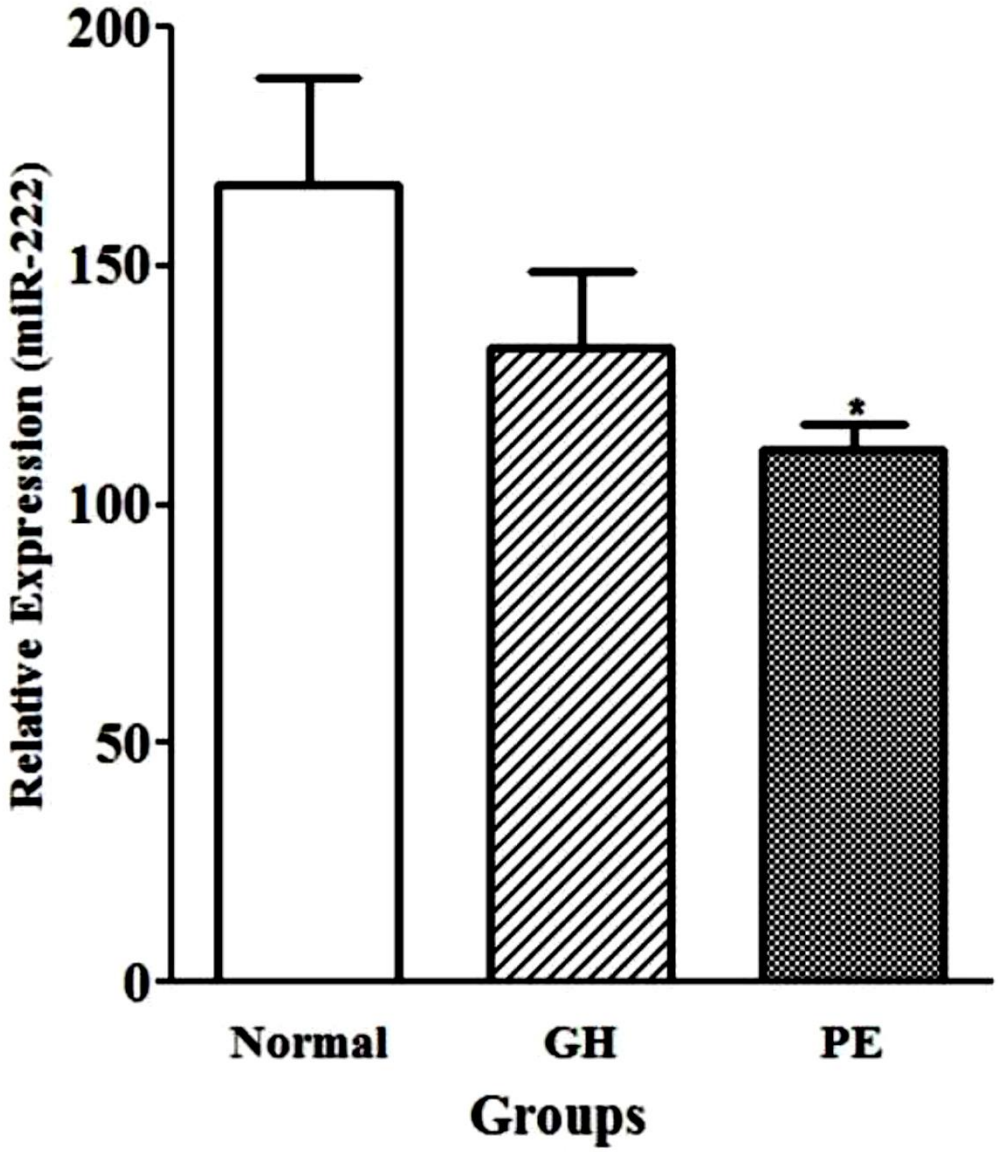


Fig.1

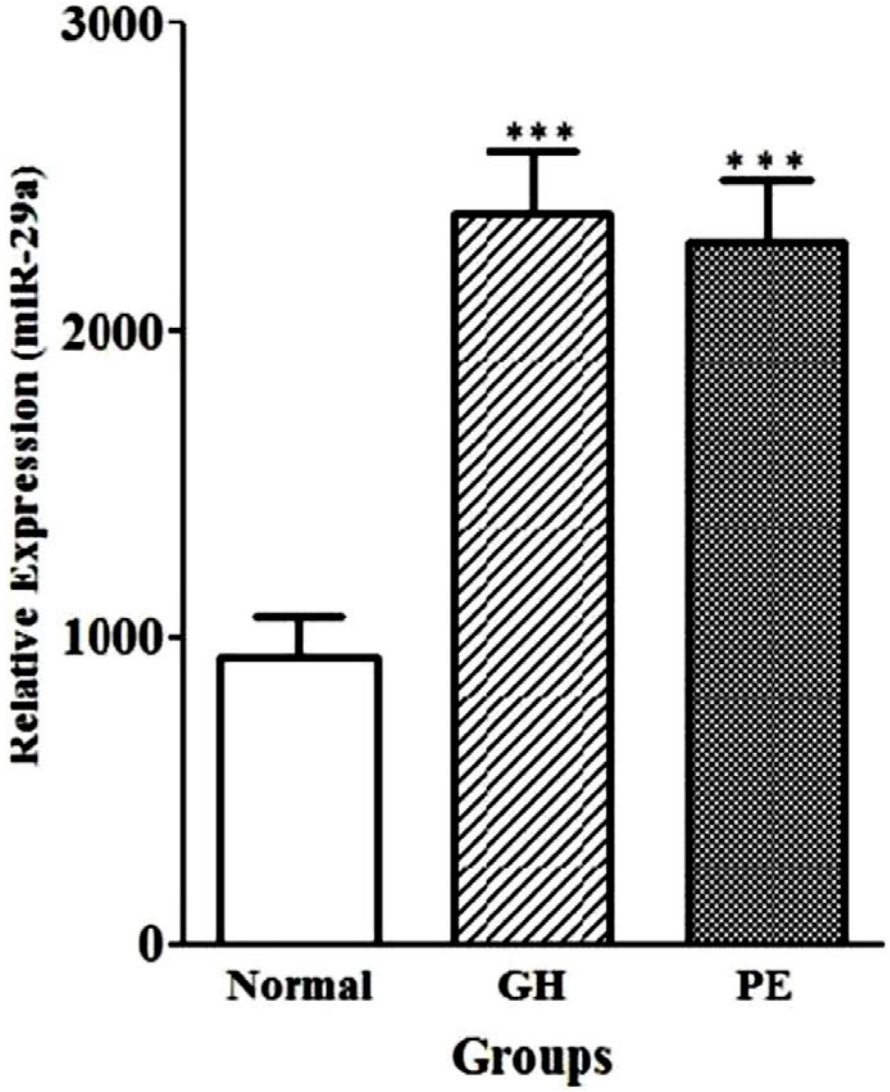

3000
2000
1000
0
Relative Expression (miR-29a)
***
***
Normal
GH
PE
Groups

Fig.2

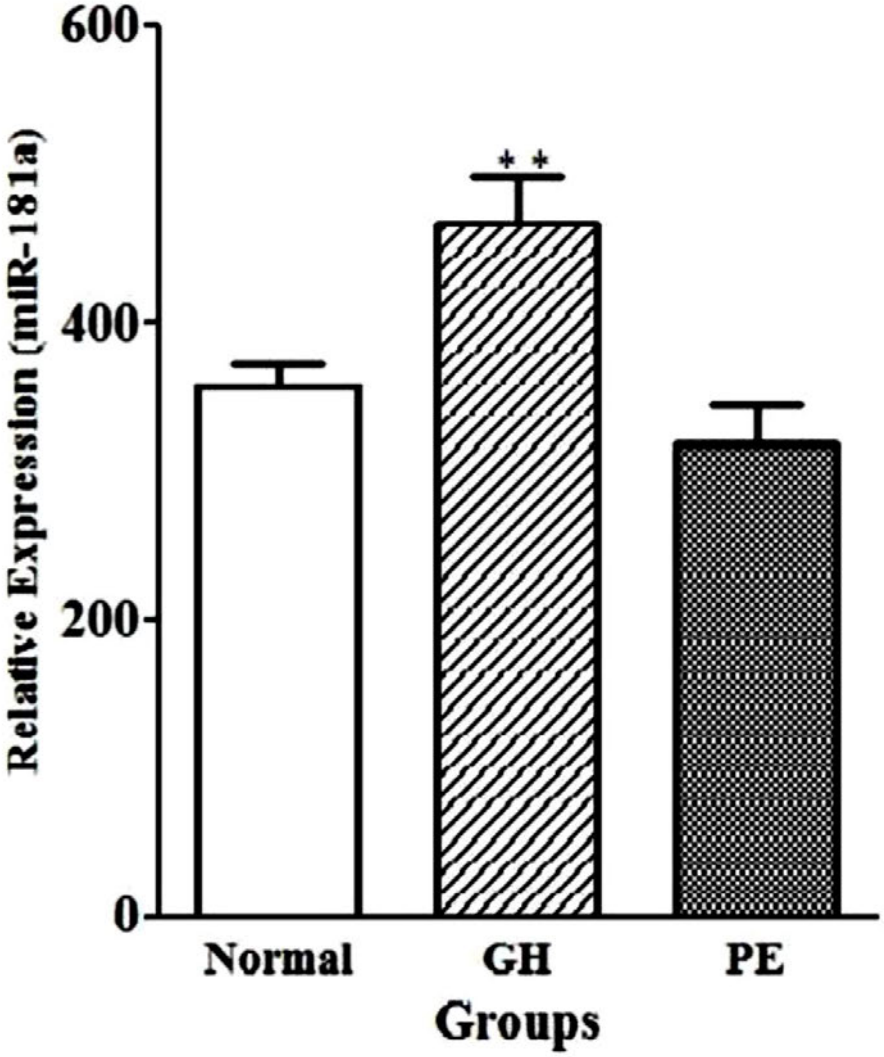

600
400
200
0
Relative Expression (miR-181a)
+ +
Normal
GH
PE
Groups

Fig.3

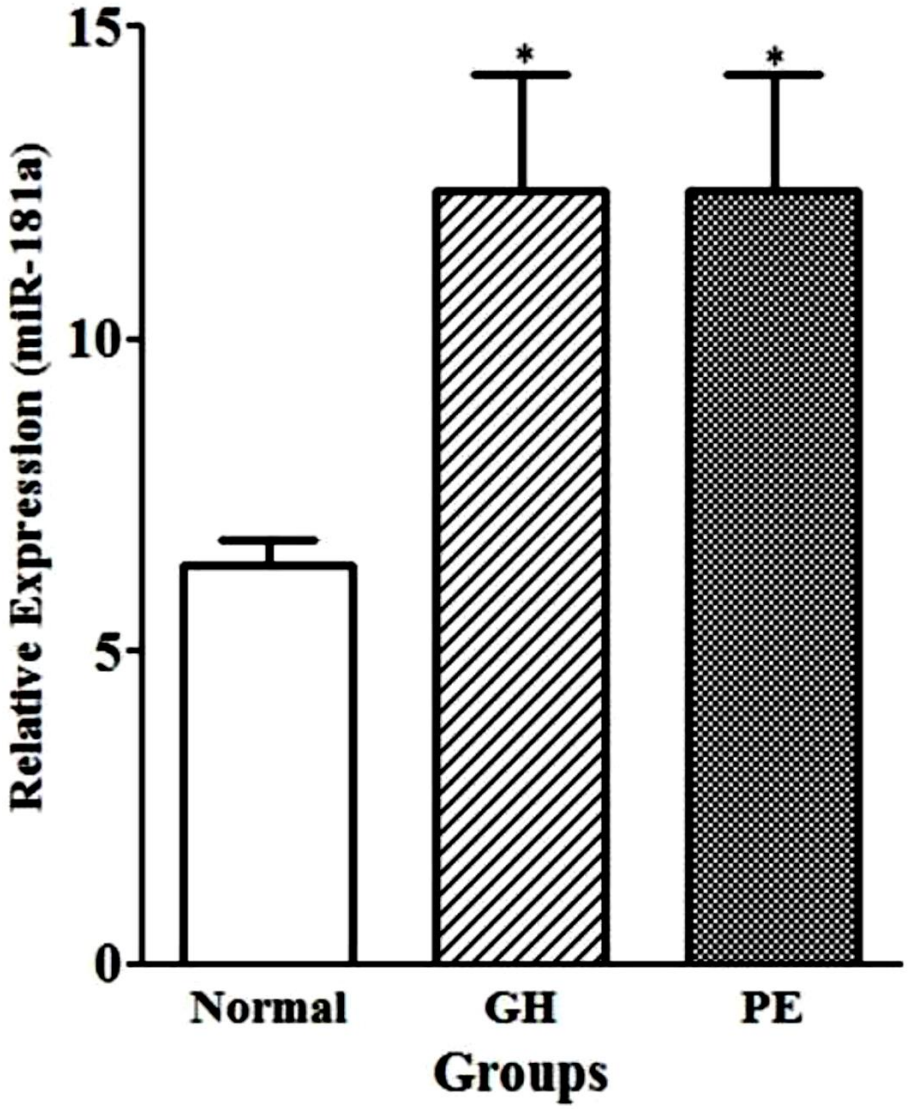
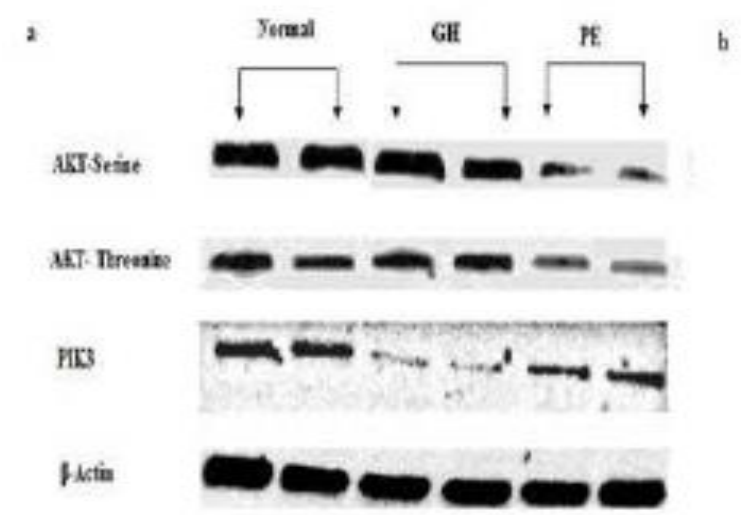
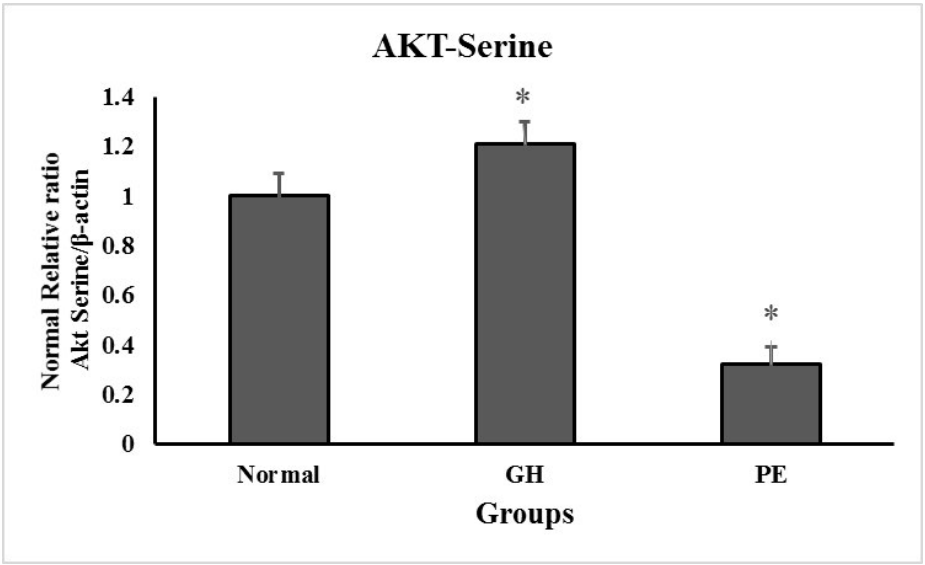
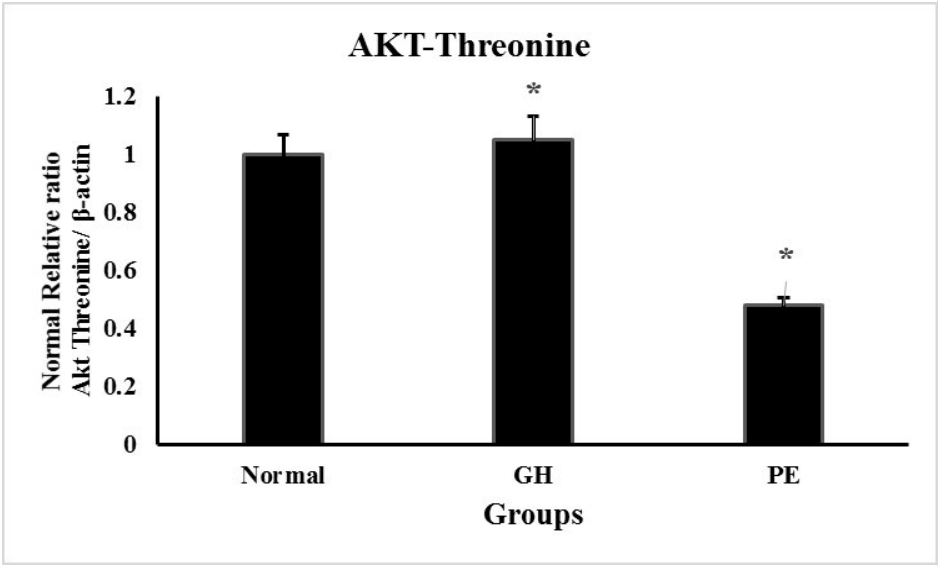


Fig.4


a
Normal
GH
PE
b
AKT-Serine
AKT- Threonine
PIK3
β-Actin


AKT-Serine
Normal Relative ratio
Akt Serine/β-actin
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Normal
GH
PE
Groups


AKT-Threonine
Normal Relative ratio
Akt Threonine/ β-actin
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Normal
GH
PE
Groups

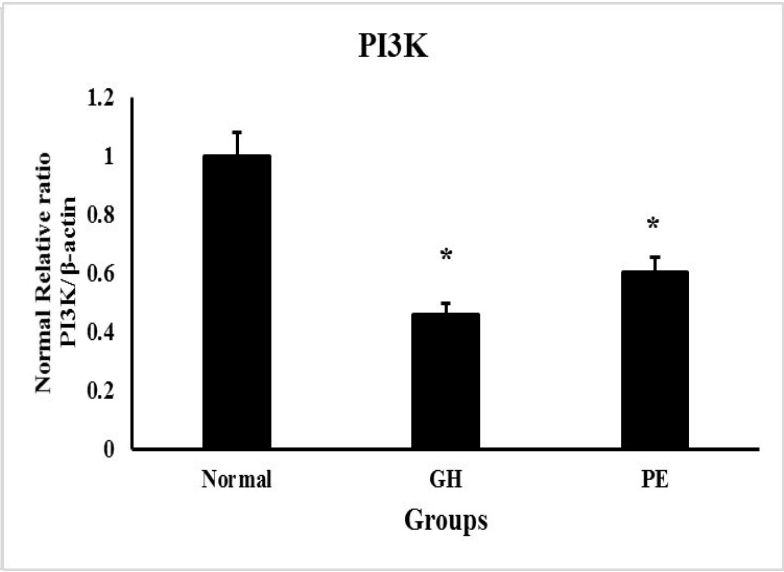

PI3K
Normal Relative ratio
PI3K/β-actin
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Normal
GH
PE
Groups

Table 1

Index	Control (n=32)	Gestational hypertension (GH) (n=28)	Preeclampsia (PE) (n=32)
Age (years)	26.8 ± 7.5	27.4 ± 7.0	29.6 ± 7.0
BMI (kg/m^2)	32.0 ± 5.7	34.2 ± 7.0	34.1 ± 9.0
Primigravidae (number)	13	12	10
Multigravidae (number)	19	16	22
Gestational age at sampling (weeks)	34.9 ± 4.2	34.1 ± 4.7	32.9 ± 4.9
Systolic blood pressure (mmHg)	108.8 ± 9.4	146.4 ±13.3*	156.7 ± 15.7#μ
Diastolic blood pressure (mmHg)	69.5 ± 9.5	97.4 ± 8.9*	102.0 ± 10.7#μ

Table 2

Name	AKT-THR	AKT-SER	PI3K
		A; NORMOTENSIVES (n=32)	
MiR-222	-1.0 **	-1.0**	-1.0**
MiR-29a	1.0**	1.0**	1.00**
MiR-181a	1.0**	1.0**	1.0**
		B; GH (n=28)	
MiR-222	1.0**	1.0**	1.0**
MiR-29a	-1.0**	-1.0**	-1.0**
MiR-181a	-1.0**	-1.0**	-1.0**
		C; PE (n=32)	
MiR-222	-1.0**	-1.0**	-1.0**
MiR-29a	-1.0**	-1.0**	-1.0**
MiR-181a	-1.0**	-1.0**	-1.0**

3 CHAPTER 3: SYNTHESIS, CONCLUSION, AND RECOMMENDATION

3.1 Synthesis

MicroRNAs are critical regulators of biological function and their role in the pathology of HDP have become a focus area (Chen and Wang, 2013; Choi *et al.*, 2013; Fu *et al.*, 2013; Pillar *et al.*, 2015; Munaut *et al.*, 2016). Studies have identified miRNAs as important biomarkers that could potentially be used as diagnostic markers in these disorders. (Hu *et al.*, 2009; Yang *et al.*, 2015; Tsochandaridis *et al.*, 2015; Jairajpuri and Almawi, 2016). PE and GH are examples of HDP with fundamental differences in their manifestations: PE patients are proteinuric while GH patients are not (Kintiraki *et al.*, 2015). However, both PE and GH are characterized by maternal endothelial dysfunction as well as metabolic dysregulations which include elevated blood pressure and insulin resistance (Poornima and Rakesh, 2014; Murphy *et al.*, 2015). It has been reported that GH may progress to PE during the course of pregnancy or in future pregnancies (Mudjari and Samsu, 2015). The present study focuses on the role of miRNAs which fundamentally affect insulin resistance, angiogenesis (placentation) and ultimately influence the clinical symptoms of these debilitating disorders. This is the first report of these miRNAs in our cohort and to the best of our knowledge, the first report in GH.

The three microRNAs (miR-222, miR-29a, and miR-181a) in the present study were chosen based on their reported role in angiogenesis and insulin resistance which researchers have shown to be altered in PE (Zhu *et al.*, 2009; Pillar *et al.*, 2015; Yang *et al.*, 2015; El-Shorafa and Sharif, 2016) using RT-qPCR. To the best of our knowledge, no studies have been conducted on miRNAs and their role in the pathogenesis of GH.

miR-222: Placental development and angiogenesis

In the present study, we found decreased expression levels of miR-222 in the serum of PE patients compared to the level in normal patients. Our findings are consistent with previous reports (Pillar *et al.*, 2015; El-Shorafa and Sharif, 2016). There was no difference in miR-222 level in GH compared to the normotensive group. miR-222 has been reported to play critical role in decidualization and implantation due to its association with endometrial stem cell differentiation. Decidualization/differentiation is vital for uterine implantation of the blastocyst, crucial for invasion of the placenta by the trophoblast cells and also placental development. Downregulation of miR-222 inhibits endometrial cell differentiation (Qian *et al.*, 2009). In addition, miR-222 is also associated with estradiol secretion, which plays an important role in early development of the placenta (Sang *et al.*, 2013). An increase in levels of miR-222 expression has been suggested to subsequently result in an increase in estradiol secretion, whereas a downregulation in miR-222 causes a decrease in estradiol secretion (Sang *et al.*, 2013). Hence, the downregulation of miR-222 in

PE may be related to poor placental development through its role in differentiation and implantation as seen in PE. However, miR-222 expression in GH did not differ from the normal group.

miR-29a: Insulin resistance and angiogenesis

Our findings also show that miR-29a was upregulated in serum in PE and GH patients compared to the normal group. These results are in agreement with that of by Geering *et al*., (2007) and Pillar *et al*., (2015). The upregulation of miR-29a has been reported to indirectly target AKT (key insulin signaling protein) by targeting PI3K, specifically its regulatory subunit (p85α) (Geering *et al.*, 2007). Increased miR-29a expression upregulates p85α, which in turn downregulates AKT (Geering *et al.*, 2007). We also found a downregulation placental AKT in PE compared to the normal group, this is in keeping with the findings of Cudmore *et al*., (2011). Interestingly, inactivation of AKT results in increased levels of soluble endoglin (sEng) a key player involved in PE pathogenesis. Soluble endoglin is an anti-angiogenic factor that inhibits the action of the vascular endothelial growth factor (VEGF) (Rădulescu *et al.*, 2016). The vascular endothelial growth factor is well known to be involved in angiogenesis but is also required for the P13K activation (Gerber *et al.*, 1998). Vascular endothelial growth factors play a role in angiogenesis through some of the metabolic pathways. Research has also shown that the addition of PI3K inhibitors affect cellular growth and results in cellular apoptosis, indicating that VEGF cannot perform its physiological function without PI3K activation (Gerber *et al.*, 1998). In the presence of VEGF and active PI3K, endothelial cell death was found to decrease. Activation of PI3K leads to AKT activation (Gerber *et al.*, 1998) and it was reported that AKT activation decreases sEng in endothelial cells (Cudmore *et al.*, 2011). Therefore, the observed upregulation of miR-29a in the present study might be associated with vascular dysfunction and insulin resistance in PE and GH.

miR-181a; Endothelial dysfunction and Insulin resistance

In the current study, the serum levels of miR-181a showed no difference in PE compared to the normal group, but there was an upregulation in GH compared to normal. In other studies, upregulation of miR-181a was found in plasma samples of PE groups between 37-40 weeks of gestation in comparison to the normal group (Wu *et al.*, 2012; Murphy *et al.*, 2015). Placental miR-181a was upregulated in the both PE and GH compared to the normal group. Our results are in keeping with several reports (Enquobahrie *et al.*, 2011; Ishibashi *et al.*, 2012; Wang *et al.*, 2012; Luo *et al.*, 2014; Xu *et al.*, 2014; Pillar *et al.*, 2015). However, some authors have reported a downregulation of this miRNA in placentae of preterm mothers with PE compared non-hypertensive mothers (Mayor-Lynn *et al.*, 2011; Choi *et al.*, 2013; Xu *et al.*, 2014)

Increased placental miR-181a expression has been observed to increase cytokine IL-6 levels, which is associated with the ATA-AA gene that activates the production of anti-angiogenic factors including sFlt-1 and sEng. (Liu *et al.*, 2012). It is well known that these factors inhibit angiogenesis in PE. Moreover, the involvement of miR-181a with the insulin signaling pathway has been shown by Wang *et al.*, (2011). Increased expression levels of miR-181a decreases the expression of the silent mating type information regulation 2 homologs-1 (Sirt-1) in mice with diabetes (Wang *et al.*, 2011). Sirt-1 deficiency has been reported to deactivate AKT signaling, leading to a conclusion that miR-181a disrupts the insulin signaling pathway in GH (Zhou *et al.*, 2012; Wang *et al.*, 2011). Results of the present study suggest that the up-regulation of miR-181a found in GH and PE may be associated with insulin resistance and endothelial dysfunction. Figure 7 below is a summary of the expression levels of miR-222, miR-181a and miR-29a in PE and GH.

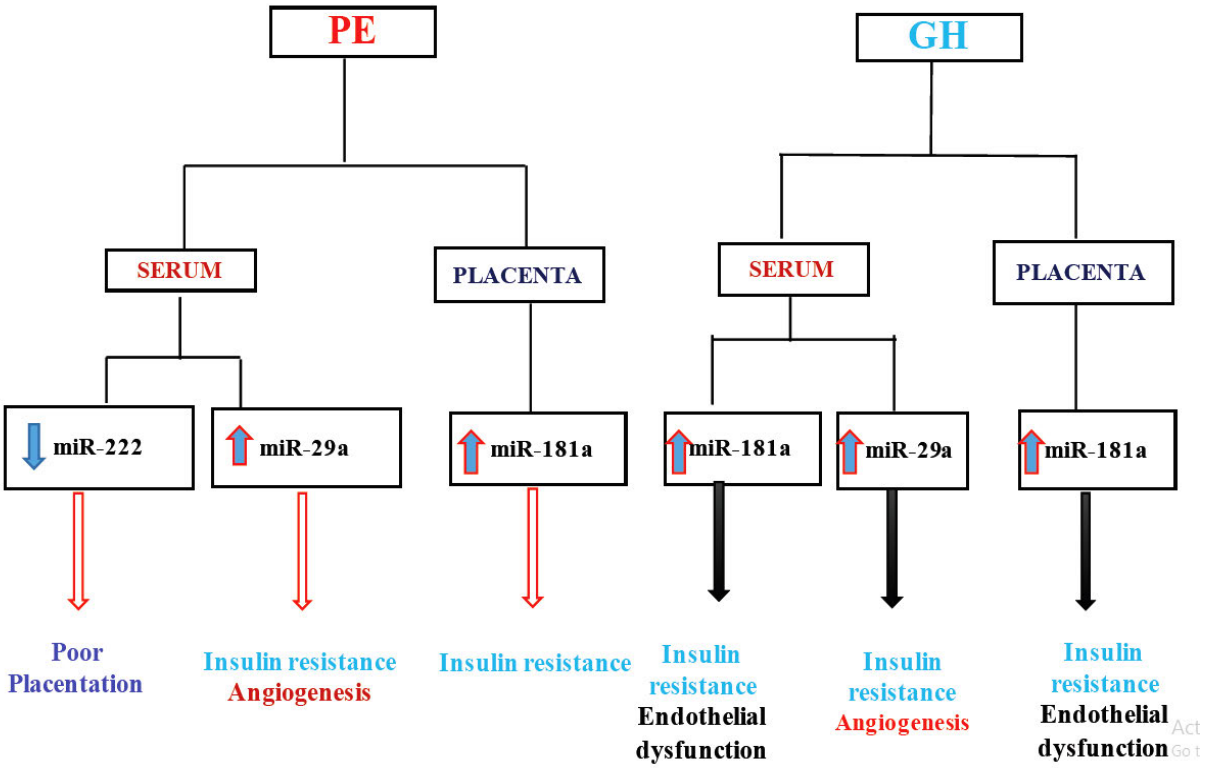


Figure 7: **Diagrammatic presentation of miRNA expression levels in GH and PE**. The three miRNAs were showed differences in their expression where a significant downregulation of miR-222 in serum samples was observed in PE but not GH groups compared to normal, thus contributing to poor placentation in PE. miR-181a was also upregulated in serum samples of GH but not PE. The similarities in miRNA expression were found in the serum expression levels of miR-29a (upregulated) and placental miR-181a (upregulated) in both PE and GH. A dysregulation of miR-29a may be associated with insulin resistance and angiogenesis in PE and GH, while a dysregulation in miR-181a may play a role in insulin resistance and endothelial dysfunction in PE and GH.

Correlation analysis

Correlation analysis was performed between the miRNAs (gene expression in placenta) and the key proteins (AKT and PI3K) in the insulin signaling pathway. A strong positive correlation between placental miR-29a, miR-181a in normotensives and the AKT/PI3K was observed ($r = 1.0, p < 0.01$). However, miR-222 showed a strong negative correlation with the proteins. The analysis showed that miR-29a and miR-181a in GH and PE groups are inversely related to the proteins (AKT and PI3K) ($r = -1.0, p < 0.01$), which would suggest that a dysregulation in these miRNAs may be associated with altered protein expression levels. miR-222 showed a strong positive correlation in the GH group with the proteins, this may be because this miRNA showed no significant difference in expression compared to normal and may therefore not play a role in the pathogenesis of GH. Our results have shown different expression levels of the three miRNAs between PE and GH. This may lead to differences in pathology.

In the present study, correlation analysis was also performed on serum and placental miRNAs because of the easy access to blood samples for diagnostics and biomarker studies. A positive correlation was observed between the placenta and serum expression levels of miR-222 in the normal group. However, placental miR-29a showed no relationship with serum miR-29a in normotensives. Placental miR-181a was inversely related to serum miR-181a in the normotensives. An inverse relationship between placental miR-222 and miR-181 with serum miR-222 and miR-181a was observed in GH and PE. A positive correlation was observed between placental miR-29a and serum miR-29a. These results show that serum samples may be used to detect a dysregulation in the placenta of GH and PE patients.

3.2 Conclusions

We found that miRNAs play an important role in the pathophysiology of pregnancy hypertensive disorders including GH. The novel findings of this study were that miR-222, miR-29a, and miR-181a are involved in the pathogenesis of GH. miR-222, miR-29a, and miR-181a are associated with metabolic dysfunction, angiogenesis and placentation in PE and GH. In particular, an upregulation of miR-181a and miR-29a in the serum samples of women with PE and GH compared to normal. Our study revealed that there is a downregulation of miR-222 in PE, compared to the normal and GH groups. These results indicate that expression levels of these miRNAs are dysregulated in PE and GH, and hence this supports the use of circulating miRNAs as potential biomarkers for non-invasive diagnosis of hypertensive disorders of pregnancy. It also reinforces the differences in the pathology of the two disorders. Furthermore, miRNAs could be targeted using novel therapeutic approaches to alter expression levels of key pathways in these disorders.

3.3 Recommendations

Several studies have been conducted to clarify the role of miRNAs in PE but no studies have been conducted in GH.

- The majority of hypertensive research has been conducted on > 20 weeks of gestation cohorts, but not much has been done in earlier weeks of gestation (< 20 weeks) to try and investigate when and how dysregulation of miRNA expression occurs. Therefore, case-control studies could be done, where pregnant women are recruited from the first antenatal visit and enrolled in the study throughout the period of gestation to delivery. With this, changes in miRNA expression levels of patients who develop PE and GH can be monitored in circulation throughout pregnancy and compared to patients with normal pregnancies.
- Research on the involvement of miRNAs in gestational hypertension and the progression of gestational hypertension into preeclampsia is scanty. Thus, further research investigating these areas are urgently warranted.

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5 APPENDIX A

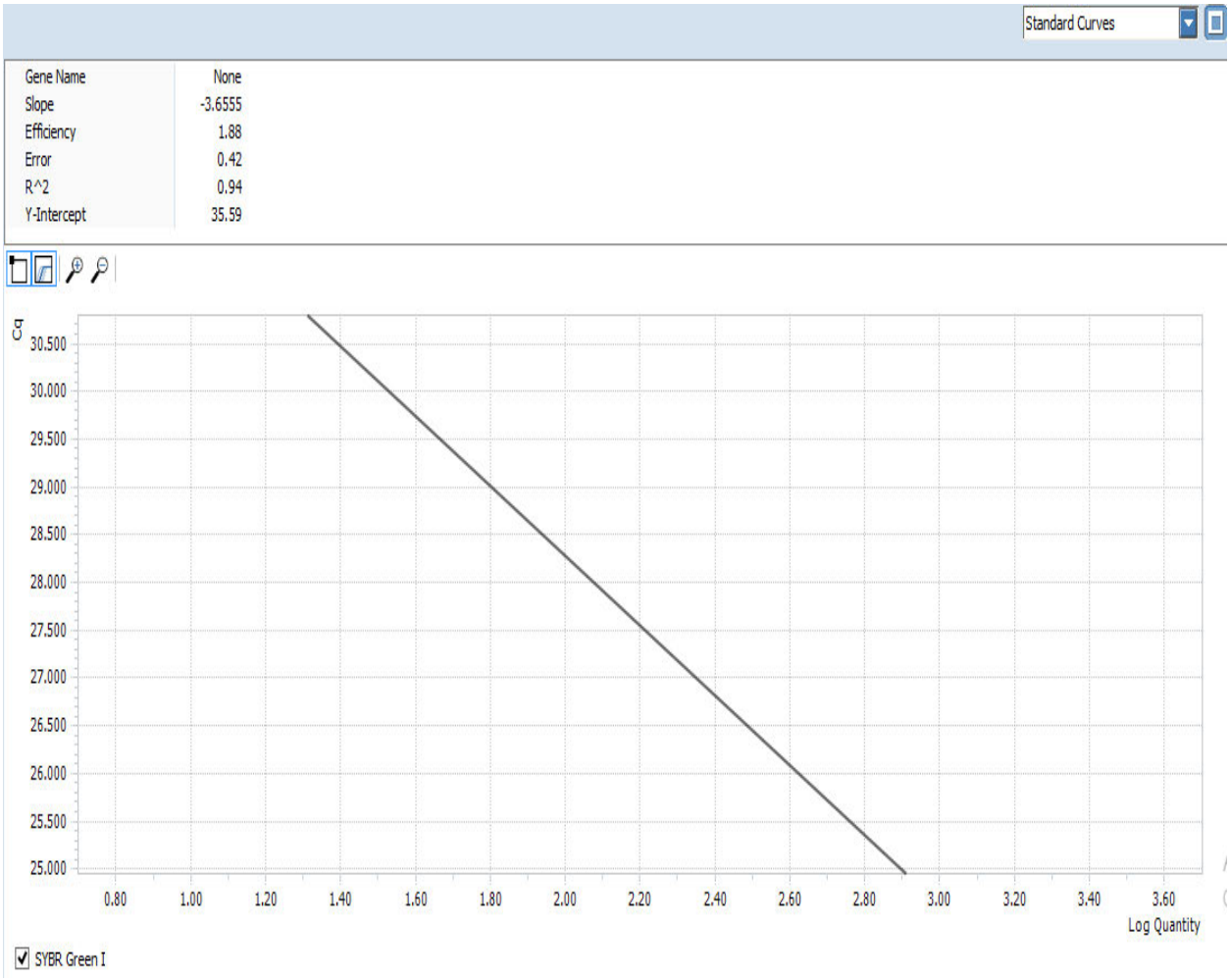

Figure A1. Standard curve using U6 small nuclear RNA (RNU6) to determine the concentration of miR-222, miR-29a, and miR-181a in serum and placental tissue.

6 APPENDIX B

Research approval from the Kwazulu-Natal Department of health

health
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DIRECTORATE:
Health Research & Knowledge Management (HKRM)

Reference: **HRKM180/16**
KZ_2016RP47_379

22 June 2016

Dear Olive Saminah Khaliq
(University of KwaZulu-Natal)

Subject: Approval of a Research Proposal

1. The research proposal titled **'The Investigation of expression levels of miR-29a, miR-181a, and miR-222 in Preeclamptic patients with Gestational Diabetes'** was reviewed by the KwaZulu-Natal Department of Health (KZN-DoH).

   The proposal is hereby **approved** for research to be undertaken at Prince Mshiyeni Memorial Hospital.

2. You are requested to take note of the following:
   a. Make the necessary arrangement with the identified facility before commencing with your research project.
   b. Provide an interim progress report and final report (electronic and hard copies) when your research is complete.

3. Your final report must be posted to **HEALTH RESEARCH AND KNOWLEDGE MANAGEMENT, 10-102, PRIVATE BAG X9051, PIETERMARITZBURG, 3200** and e-mail an electronic copy to hrkm@kznhealth.gov.za

For any additional information please contact Ms G Khumalo on 033-395 3189.

Yours Sincerely

[redacted]

Chairperson, Health Research Committee
Date: 22/06/16.

Fighting Disease, Fighting Poverty, Giving Hope

7 APPENDIX C

Ethical Approval from the University of Kwazulu-Natal Biomedical Research Ethics Committee (BREC)

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

05 July 2016

Mrs OPS Khaliq
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Protocol: The investigation of expression levels of miR-29a, miR-181a, and miR-222 in preeclamptic patients with gestational diabetes.
Degree: MMedSc
***BREC reference* number: BE229/16**

EXPEDITED APPLICATION

The Biomedical Research Ethics Committee has considered and noted your application received on 31 March 2016.

The study was provisionally approved pending appropriate responses to queries raised. Your response received on 28 June 2016 to queries raised on 08 June 2016 have been noted and approved by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given **full ethics approval.**

This approval is valid for one year from **05 July 2016.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.
BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **RATIFIED** by a full Committee at its meeting taking place on **16 August 2016.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

[redacted] Ethics Committee

[redacted].ac.zazn.ac.za cc postgraduate office: tarinm@ukzn.ac.za

8 APPENDIX D

Amendment letter of Ethical Approval from the University of Kwazulu-Natal Biomedical Research Ethics Committee (BREC).

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI

RESEARCH OFFICE
BIOMEDICAL RESEARCH ETHICS ADMINISTRATION
Westville Campus
Govan Mbeki Building
Private Bag X 54001
Durban
4000
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 260-4609
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

Amended letter
11 August 2016

Mrs OPS Khaliq
School Of Laboratory Medicine and Medical Sciences
Health Sciences
216074799@stu.ukzn.ac.za
saminah.khaliq@gmail.com

Old Title of Protocol: The investigation of expression levels of miR-29a, miR-181a, and miR-222 in preeclamptic patients with gestational diabetes.
Degree: MMedSc
BREC reference number: BE229/16

New Title of Protocol : The investigation of miR-29a, miR-181a, and miR-222 in preeclampsia and gestational hypertension.

Your correspondence received 27 July 2016 submitting an Application for Amendments to replace gestational diabetes group with gestational hypertension and change of the original title for the above study has been noted and approved by a sub-committee of the Biomedical Research Ethics Committee.

This approval will be ratified at a full BREC meeting taking place on 13 September 2016.

Yours sincerely

[redacted]

Senior Admin Officer: Biomedical Research Ethics Committee

cc supervisor: mackraj@ukzn.ac.zazn.ac.za cc postgrad: berinm@ukzn.ac.za

9 APPENDIX E

Prince Mshiyeni Memorial Hospital approval letter to conduct research

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

DIRECTORATE: Senior Medical Manager

Mangosuthu Highway, Private Bag X 07
MOBENI
Tel: 031 907 8317/8304 Fax: 031 906 1044 Email:myint.aung@kznhealth.gov.za
www.kznhealth.gov.za

Prince Mshiyeni Memorial Hospital

Enquiry: Dr M AUNG
Ref No: 11/RESH/2106
Date: 31/05/2016

TO: Olive Khaliq

RE: LETTER OF SUPPORT TO CONDUCT RESEARCH AT PMMH

Dear researcher;

I have pleasure to inform you that PMMH has considered your application to conduct research on **"The Investigation of expression levels of miR-29a, miR-181a, and miR-222 in Preeclamptic patients with Gestational Diabetes"** in our institution.

Please note the following:

1. Please ensure that you adhere to all the policies, procedures, protocols and guidelines of the Department of Health with regards to this research.
2. This research will only commence once this office has received confirmation from the Provincial Health Research Committee in the KZN Department of Health.
3. Please ensure this office is informed before you commence your research.
4. The institution will not provide any resources for this research.
5. You will be expected to provide feedback on you finding to the institution.

Should the following requirements be fulfilled, a Permission/ Approval letter will follow.

- Full research protocol, including questionnaires and consent forms if applicable.
- Ethical approval from a recognized Ethic committee in South Africa

Thank you.

[redacted]

MYINT AUNG
Senior Medical Manager & specialist in Family Medicine
MBBS, DO(SA), PGDip in HIV (Natal), M.Med.Fam.Med (natal)
Tel: 031 9078317
Fax: 031 906 1044
myint.aung@kznhealth.gov.za

10 APPENDIX F

DATA SHEET: To investigate the expression levels of miR-29a, miR-181a, and miR-222 in Preeclamptic and Gestational hypertension

Study site: PMMH **Study number: ……**

Study groups: Normotensive, Preeclampsia, Gestational hypertension

GENERAL HOSPITAL INFORMATION:

Hospital number

PATIENT DEMOGRAPHICS:

Age	Rural dweller	Urban dweller	Life style

Clinical Data

Patient Demographics	**Status**	**Disorders**
Gravidity		
Parity		
Gestational age		
Maternal Height		
Maternal weight		
Systolic blood pressure		
Diastolic blood pressure		
Proteinuria		

11 APPENDIX G

Patient Consent

Dear Ms, Miss, Mrs:

I am Olive Khaliq. I am doing a research project for my higher degree (MMEDSc) at the University of KwaZulu-Natal. I would like your permission to obtain 10 mls (1 tablespoon) of blood from your arm and placenta after birth. The taking of blood will not cause you any pain but a little bit of discomfort. The blood sample will be used to extract serum for microRNA detection. For this study I will use two methods of testing which are a real time PCR as well as Western blot analysis. For Western Blot, I will need to use placental tissue, this is to investigate whether the microRNAs have an effect on the proteins involved in controlling blood sugar. With these two methods I will try to see the expression levels of microRNAs in preeclampsia and Gestational hypertension. This test may be helpful in the future in predicting whether Gestational Hypertension can lead to preeclampsia due to these miRNAs.

Please note that I am doing a study for my higher degree and may publish the findings, but your identity will not be disclosed. All the information will be strictly confidential and any blood samples and tissues not used will be destroyed.

Please do not hesitate to let me know if you would like more information. Note also that your participation in this research will not affect your treatment/management in any way.

Thank You

Patient file no……..

Patient Signature:_____ Date:_____

Sawubona Nks/ NKK

I gama lami ngingu- Olive Khaliq ngenza ucwaningo lwemfundo ephakeme kwiziqu zesayensi yokwelapha (MMEDSc) eNyuvesi yaKwaZulu- Natal. Ngingathanda ukuthola imvume yakho yokuthola 10 mls (isipuni esisodwa) segazi lakho elizodonswa engalweni nakwiplasenta (placenta) emvakokubeletha. Ukuthathwa kwegazi akuzokuzwisa ubuhlungu obukhulu ngaphandle nje kokuthi kuzokwenza ungakhululeki kancane. Izicubu zegazi zizosetshenziswa ukukhipha i serumu (serum) ukuze kuhlolwe i microRNA. Kulesifundo ngizosebenzisa izindlela ezimbili zokuhlola okuyi real time PCR Kanye ne western blot. Ngokucwaninga ngisebenzisa i western blot ngizodinga ukusebenzisa izicubu zeplasenta (Placenta). Kulokhu ngizobe

ngifuna ukuthola ukuthi angabe ama microRNAs anawo yini umthelela kwizakhamzimba ezibandakanyeka ekulawuleni ushukela egazini. Ngalezindlela ezimbili zokuhlola ngizozama ukubona ubukhona bezinga lama microRNA kwi sifo esibizwa nge preeclampsia Kanye nesifo sebhiphio etholakala kwabakhulelwe (Gestational hypertension). Locwaningo lungaba usizo kusasa ekuqaguleni ukuthi ibhiphi etholakala kubantu abakhulelwe (gestational hypertension) ingaholela kwisifo esibizwa nge preeclampsia ngenxa yokukhuphuka kwalama miRNAs.

Ngiyacela ungangabazi ukungazisa uma ufuna ulwazi oluthexaxa. Qaphela nokuthi ukuzibandakanya kwakho kulocwaningo akuzothikameza indlela imishanguzo yakho esebenza ngakhona nanoma ngayiphi indlela.

Ngiyabonga

Inamba fayela yesiguli……..

Ukusayina kwesigulu……
Usuku……..