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The effect of placental growth hormone in pregnancy

Shutan Liao

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Health Sciences,
The University of Auckland, 2017
Outputs arising from this thesis

Published papers

Appendix I, II, III:


Manuscripts in submission


Abstract

Background: The growth hormone (GH) and insulin-like growth factor-1 (IGF-1) axis is a major regulator of mammalian growth. In humans, two GH genes encode two GH proteins: pituitary GH (GH-N) and GH-V variant (GH-V). GH-V is thought to play a key role in maternal adaptation to pregnancy. However, to date, the exact function of GH-V during pregnancy is poorly understood.

Aim: The primary aim of the work detailed in this thesis was to identify the effect of GH-V on pregnancy outcomes, in terms of fetal growth and maternal metabolism, in experimental animal models and to determine whether circulating GH-V was altered in a human cohort with well characterized pregnancy outcomes.

Methods:

1) Literature review: In order to determine the gap in research knowledge, Pubmed, Medline and CINAHL databases were searched for literature related to placental development, and potential roles of GH in normal and pathological pregnancies.

2) An animal study was conducted to determine the dose-response relationship for human GH-V treatment in a mouse model of normal pregnancy. Following time-mating, pregnant wild-type C57BL/6J mice were randomized to receive different doses of GH-V treatment (0.25, 1, 2, 5 mg/kg per day) or vehicle control from gestational day 12.5 to 18.5 via osmotic pump.

3) Daily versus continuous administration of GH-V was compared in non-pregnant mice to determine the effect of GH-V on mice via different modes of administration. C57BL/6J female mice were randomized to receive vehicle or GH-V (2, 5 mg/kg per day) via injection or osmotic pump for 6 days.

In both animal studies body weight and food intakes were recorded regularly. Blood samples and tissue of interest for each independent experiment were collected at different time points.
4) Nested case-control studies: pregnancies associated with inappropriate weight for gestational age, gestational diabetes mellitus (GDM) and pre-eclampsia (PE) were selected from the Screening for Pregnancy Endpoints (SCOPE) study in Auckland, and matched to control cases. Maternal serum samples taken at 20 weeks were assayed for concentrations of GH-V, insulin-like growth factors (IGF-1 & 2) and their major binding proteins (IGFBP-1 & 3) using an enzyme-linked immunosorbent assays (ELISAs) developed in-house.

**Results:** In mouse models, GH-V treatment during pregnancy did not affect maternal body or fetal body weights. However, treatment with 5 mg/kg per day significantly increased maternal fasting plasma insulin concentrations with impaired insulin sensitivity observed at day 18.5. Further, different modes of GH-V delivery had differential effects on growth and metabolism. Compared to continuous treatment, pulsatile GH-V treatment in non-pregnant mice was more effective in stimulating growth but caused marked hyperinsulinemia in mice. In human studies, maternal serum GH-V concentrations in large-for-gestational age (LGA) pregnancies were significantly higher than in appropriate-for-gestational age (AGA) and small-for-gestational age (SGA) pregnancies. Maternal GH-V concentrations at 20 weeks of gestation were positively correlated to birth weight but were not altered in pregnancies complicated by GDM or PE.

**Conclusions:** The findings suggest that maternal GH-V is associated with fetal growth and is a likely mediator of the insulin resistance in pregnancy, implicating a unique role for GH-V in normal and pathological pregnancies.
Acknowledgements

All that I have accomplished during my PhD years would not have been possible without the guidance, encouragement and support of many wonderful people. I would like to take this opportunity to express my appreciation and to acknowledge these individuals, to whom I am greatly indebted. First and foremost, I would like to express my sincere gratitude to my supervisors, Dr Jo K Perry and Professor Philip N Baker – for their keen scientific training, steadfast guidance and mentorship, and on a personal level, for being incredibly supportive and understanding throughout my PhD adventures. I would also like to thank my supervisory committee members Associate Professor Mark H Vickers, Dr Joanna L Stanley and Dr Anna Ponnampalam for their scientific guidance, experimental advice, helpful criticism and honest commitment.

Throughout the years, I have had the opportunity to work with some wonderful colleagues at the Liggins. Thank you all for your unwavering support, stimulating discussions and technical guidance. I would like to express my gratitude to all the funding sources for the work contained in my thesis. Funding for this work was provided by Gravida: National Centre for Growth and Development. Also, thanks to Gravida for providing funding for travel awards.

Finally, I would like to express my heartfelt thanks and appreciation to my family—my amazing parents and my wife.
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<tbody>
<tr>
<td>Ach</td>
<td>acetycholine</td>
</tr>
<tr>
<td>AGA</td>
<td>appropriate for gestational age</td>
</tr>
<tr>
<td>AKT</td>
<td>v-akt murine thymoma viral oncogene</td>
</tr>
<tr>
<td>ALS</td>
<td>acid labile subunit</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>COMT</td>
<td>catechol-O-methyl transferase</td>
</tr>
<tr>
<td>CS</td>
<td>chorionic somatomammotropin</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>d3-GHR</td>
<td>exon3-deleted growth hormone receptor</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>EVT</td>
<td>extravillous trophoblast</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FGR</td>
<td>fetal growth restriction</td>
</tr>
<tr>
<td>fl-GHR</td>
<td>full-length growth hormone receptor</td>
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<tr>
<td>GDM</td>
<td>gestational diabetes mellitus</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>GH1</td>
<td>pituitary growth hormone</td>
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<tr>
<td>GH2</td>
<td>placental growth hormone variant</td>
</tr>
<tr>
<td>GHBP</td>
<td>growth hormone binding protein</td>
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<tr>
<td>GHIH</td>
<td>growth hormone-inhibiting hormone</td>
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<tr>
<td>GH-N</td>
<td>pituitary growth hormone</td>
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<td>GHR</td>
<td>growth hormone receptor</td>
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<td>GHRH</td>
<td>growth hormone-releasing hormone</td>
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<td>GH-V</td>
<td>placental growth hormone variant</td>
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<tr>
<td>GLUT</td>
<td>glucose transporter</td>
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<tr>
<td>hCG</td>
<td>human chorionic gonadotropin</td>
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<td>ICM</td>
<td>inner cell mass</td>
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<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
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<td>IGFBP</td>
<td>insulin-like growth factor binding protein</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>INSR</td>
<td>insulin receptor</td>
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<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
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<tr>
<td>JAK</td>
<td>Janus kinase</td>
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<tr>
<td>LCR</td>
<td>locus control region</td>
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<tr>
<td>LGA</td>
<td>large for gestational age</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
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<tr>
<td>OD</td>
<td>optical density</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>OGTT</td>
<td>oral glucose tolerance test</td>
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<td>PE</td>
<td>pre-eclampsia</td>
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<tr>
<td>PHE</td>
<td>phenylephrine</td>
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<tr>
<td>PI3-K</td>
<td>phosphoinositide 3-kinase</td>
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<tr>
<td>PL</td>
<td>placental lactogen</td>
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<tr>
<td>PPARγ</td>
<td>peroxisome proliferator-activated receptor-γ</td>
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<tr>
<td>PRL</td>
<td>prolactin</td>
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<tr>
<td>PSS</td>
<td>physiological saline solution</td>
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<tr>
<td>RIA</td>
<td>radio immunoassays</td>
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<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
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<tr>
<td>SCOPE</td>
<td>Screening for Pregnancy Endpoints</td>
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<tr>
<td>sFlt1</td>
<td>soluble Fms-like tyrosine kinase 1</td>
</tr>
<tr>
<td>SGA</td>
<td>small for gestational age</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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Chapter three of this thesis is an revised version of Shutan's first author peer-reviewed article "The Placental Variant of Human Growth Hormone Reduces Maternal Insulin Sensitivity in a Dose-Dependent Manner in C57BL/6J Mice" published in the journal Endocrinology. 2015, 157(3), pp.1175-1186.

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The undersigned hereby certify that:
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- that the candidate wrote all or the majority of the text.

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Chapter 5 Section 5.3 of this thesis consists of a revised version of Shutan's first author peer-reviewed article "Human placental growth hormone is increased in maternal serum at 20 weeks of gestation in pregnancies with large-for-gestational-age babies" which has been accepted by the journal Growth Factors, GGRF-2016-0064.

Chapter 5 Section 5.4 and 5.5 of this thesis consists of revised versions of two of Shutan's first author articles "Maternal serum placental growth hormone at 20 weeks' gestation in pregnancies complicated with gestational diabetes mellitus" and "Maternal serum placental growth hormone at 20 weeks' gestation in pregnancies complicated with pre eclampsia" which are being prepared for submission.

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<td>Designing and conducting the experiments, analysing data, critically evaluating the manuscript, responding to reviewer's comments</td>
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**Certification by Co-Authors**

The undersigned hereby certify that:
- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

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Chapter 1: Introduction

Growth hormone (GH) is a peptide hormone that stimulates growth, cell replication and cell regeneration in humans and other animals. During pregnancy, humans produce two primary versions of the GH protein: the pituitary GH (GH-N) and the GH-V variant (GH-V). GH-V, which is produced by the placenta, is thought to play a key role in the maternal adaptation to pregnancy and is associated with a number of pathological pregnancy conditions.

Chapter 1 will provide an overview of the structure and development of the placenta as well as the epidemiology and aetiology of some pathological pregnancies. Further, the potential role of GH-V in normal and pathological pregnancies will also be discussed.

1.1. Structure of the placenta

In humans, the placenta is a discoid shape of approximately 20 cm in length, 2.5 cm in thickness, and a surface area of almost 15 m². It is thickest at the centre and thinner at the edges. It typically weighs 500 grams in normal term pregnancies but this varies between different ethnicities and geographical regions. The placenta connects to the fetus by an umbilical cord, which contains two umbilical arteries and one umbilical vein.

The human placenta is a composite organ composed of both maternal and fetal tissue. The fetal part of the placenta is made up of the chorionic plate with its placental villi, the cytotrophoblast layer and the intervillous spaces; the maternal side of the placenta is made up of the decidua basalis, uterine vessels and glands (Gude et al., 2004). The basic structural unit of the placenta is the chorionic villus. The villi are vascular projections of fetal tissue surrounded by chorion. The human placenta is haemochorial,
in which maternal blood is in direct contact with the chorion (Rai and Cross, 2014). The chorion consists of two cellular layers: the outer syncytiotrophoblast and the inner cytotrophoblast. Circulating maternal blood enters the intervillous space via spiral endometrial arteries, bathes the chorionic villi, and drains back through endometrial veins, allowing an exchange of substances to take place. The placenta receives blood supplies from both the maternal and the fetal systems and thus has two separate circulatory systems for blood: the maternal-placental (uteroplacental) blood circulation, and the fetal-placental (fetoplacental) blood circulation. There is no auto-regulation in the uteroplacental circulation due to the lack of neuronal innervation and therefore blood flow is directly related to the uterine perfusion and inversely related to uterine vascular resistance (Khong et al., 1997). Some studies do suggest that local oxygen and nitric oxide (NO) levels, paracrine and circulating factors may also contribute to blood flow regulation in the placenta (Hampl et al., 2002; Rosenfeld et al., 2002; Zheng et al., 2005).

1.2. Development of the placenta

A characteristic feature of mammalian reproduction is the prolonged protection afforded to the offspring, which is quite different from ovipara where there is little or no other embryonic development within the mother. Intrauterine pregnancy marks a major evolutionary step from previous modes of reproduction and decreases morbidity and mortality dramatically. However, it requires a series of anatomic and physiologic adjustments for both the mother and the fetus. Among these adjustments, the development of the placenta is unique and makes intrauterine pregnancy possible.

The placenta is a key trait of eutherian mammals and plays a key role in fetal growth through multiple mechanisms. It is the zone of exchange for gas and nutrients between
maternal and fetal compartments, and is composed of active endocrine and neuroendocrine tissue. As an endocrine organ, the placenta produces a number of endocrine, paracrine and/or autocrine factors include oestrogens, progesterone, chorionic gonadotropin, placental lactogen, GH-V, growth factors (including epidermal growth factor, insulin-like growth factors (IGF) 1 and 2, platelet-derived growth factor, cytokines, chemokines, eicosanoids and related compounds, vasoactive autacoids, pregnancy-associated proteins of placental origin, corticotrophin-releasing hormone, gonadotrophin-releasing hormone, thyrotrophin-releasing hormone and many others (Evain-Brion and Malassine, 2003). The placenta grows throughout pregnancy and appropriate development is essential for the maternal adaptation to pregnancy and fetal growth.

1.2.1. **Blastocyst formation**

After coitus, the sperm fertilizes an ovum by fusion which results in the formation of a zygote. Following fertilization, the zygote undergoes a series of changes, including division, compaction, and the formation of desmosomes and gap junctions. As a result, a central cavity of the cell mass is formed, and blastocyst is generated. Asymmetrical division of morula cells then results in two distinct populations of cells with different cellular components: outer trophoblast cells that form the wall of the blastocyst, and the inner cell mass (ICM) that gives rise to the embryo and the placenta. Critical gene families controlling these events include the E-cadherin-catenin cell adhesion family, the tight junction gene family, the Na/K-ATPase gene family and perhaps the aquaporin gene family (Nishioka *et al.*, 2009; Watson and Barcroft, 2001). A number of growth factors, either secreted into the lumen of the female reproductive tract from maternal side or synthesized by the developing embryo itself, regulate the development of preimplantation mammalian embryos. Transforming growth factor (TGF) beta is
produced by preimplantation mouse embryos, suggesting that TGF beta may function in the preimplantation embryo development through autocrine and/or paracrine mechanisms (Chow et al., 2001; Roelen et al., 1998); the presence of epidermal growth factor (EGF) during preimplantation development increases implantation rate in rats (Aflalo et al., 2007), while Leukemia inhibitory factor and its receptor are consistently decreased at the 4-cell stage of embryonic development (Chen et al., 1999). Pantaleon et al. (Pantaleon et al., 1997) reported that GH receptor (GHR) transcripts can be detected in the preimplantation mouse embryo as early as the 2-cell stage. In sheep and cow, the GHR mRNA was also found in all stages of embryo development before implantation (Izadyar et al., 2000; Lacroix et al., 1999). Both GH and IGF-1 increased the cell number in cultured bovine embryos (Moreira et al., 2002). These observations raise the possibility of a paracrine/autocrine GH loop regulating embryonic development in its earliest stages.

1.2.2. Implantation and invasion

The implantation process begins with apposition and adhesion of the blastocyst to maternal endometrium. At this stage, the wall of the blastocyst consists of two layers: an outer layer of trophoblast cells and an inner layer of extraembryonic mesoderm derived from the ICM. At the apical junctional complexes between the trophoblast layer and the endometrium, the trophoblasts induce the lysis of the connection of the uterine epithelial cells, opening up the intercellular spaces and enabling the protrusions of trophoblast cells to proliferate and penetrate into the endometrium (Carson et al., 2000). Those trophoblast cells which penetrate and differentiate to form a multinucleated mass are called the syncytiotrophoblast while the remaining cells of the blastocyst wall remain unfused and are referred as cytotrophoblasts. A population of cytotrophoblast cells differentiates, and migrates into the endometrium. These migratory cells are
known as extravillous trophoblast (EVT), and they start the process of trophoblast invasion (Genbacev et al., 1992).

During the early stage of placentation, EVT cells penetrate the syncytiotrophoblast shell and enter the decidua and subsequently the myometrial stroma at anchoring villi (Figure 1.1). In this process, the migration of EVT forms two subpopulations. A subset of EVT cells reach the walls of spiral arteries immediately after they invade into the endometrium, and are referred to endovascular trophoblast while the others are called interstitial trophoblast cells (Kaufmann et al., 2003).
Figure 1.1: Trophoblast invasion and spiral artery remodelling. EVT cells first enter the decidua and subsequently the myometrial stroma as interstitial trophoblast. This encircles and destroys the smooth muscle cells of spiral artery media which is replaced by amorphous fibrinoid material. Subsequently, endovascular trophoblasts invade the lumen of the arteries to replace the endothelium of the vessels.

Spiral arteries are small arteries which temporarily supply blood to endometrium of the uterus during the luteal phase of the menstrual cycle. During pregnancy, the uterine spiral arteries are progressively remodelled to form dilated conduits lacking maternal vasomotor control. Adequate remodelling of maternal uterine spiral arteries is crucial for a healthy pregnancy. These physiological changes of utero-placental arteries can be divided into two stages: trophoblast-independent transformation and trophoblast-dependent transformation (Smith et al., 2009). The spiral arteries undergo a series of
changes prior to the arrival of the trophoblast cells, and these changes include endothelial basophilia and vacuolation, disorganized vascular smooth muscle cells, increased endothelial activation and vessel dilation (Craven et al., 1998). The conversion of a tonic maternal arteriole composed of multiple layers of vascular smooth muscle, elastin and numerous other extracellular matrix components, into a highly dilated yet durable vessel, requires tight regulatory control and the coordinated actions of multiple cell types. Placental development is not only reliant on the fetal trophoblast but also the maternal cells present in the decidua. Uterine natural killer (NK) and EVT cells are proposed to be the key regulators of spiral artery remodelling (Tessier et al., 2015). Recent evidence also suggests that the initiation of the remodelling process is carried out primarily by uterine NK cells, while the EVT cells play more of a secondary role (Charalambous et al., 2012; van der Heijden et al., 2005). The exact function of NK cells in arterial remodelling is not fully understood. However, they appear to be involved in the degradation and clearance of the spiral arterial smooth muscle cell layer and endothelial cells, which allows dilatation of the spiral arteries (Ashkar et al., 2000; Caluwaerts et al., 2005). As the remodelling of arterial components occurs, EVT cells migrate and replace the degraded endothelial cells and line the spiral arterial walls (Wallace et al., 2012). The EVT cells achieve this through two different migratory pathways. The endovascular trophoblast cells migrate along the arterial lumen retrograde to the blood flow by adhering to and replacing the endothelium, forming intraluminal trophoblastic plugs. The interstitial trophoblasts invade within the vascular wall tissues in the decidua as far as the inner third of the myometrium (Knofler, 2010). The interaction of EVT with the spiral arteries results in specific changes to vessel wall structure with apoptosis, destruction of the extracellular matrix and induction of vascular smooth muscle cell de-differentiation (Desforges et al., 2015).
The number of invading trophoblast cells as well as the depth and width of invasion has profound influence on feto-uterine circulation. A spectrum of placenta and pregnancy pathologies, such as fetal growth restriction (FGR) and pre-eclampsia (PE), are linked to the failure or inadequate trophoblast invasion, and spiral artery remodelling. In FGR and PE cases, a reduction in the depth of invasion and in the number of invaded arteries can be observed (Kadyrov et al., 2006). In severe FGR, typically a smaller placenta and the eccentric cord can also be seen due to the early loss of a large part of the chorion with the insufficiency of invasion (Viero et al., 2004). In addition to FGR, inappropriate trophoblast invasion, fewer invasive trophoblasts and failure to remodel the spiral arteries are also found in the placental pathology of PE (Lyall et al., 2013; Naicker et al., 2003).

An increasing number of growth factors, cytokines and angiogenic molecules controlling trophoblast motility have been identified. These factors are secreted from numerous cells such as trophoblast, maternal epithelial and stromal cells, as well as uterine NK cells and macrophages, suggesting that a complex network of cell types, mediators and signalling pathways regulate trophoblast invasiveness. Karmakar and Das (Karmakar and Das, 2002) found that interleukin (IL)-1beta and TGF-beta1 seemed to be critical for regulating the protease network thereby effectively controlling the trophoblast invasion into the endometrium. EGF plays an important role by up-regulating trophoblast invasion during the early stages of pregnancy (Bass et al., 1994). The presence of IGFs at the maternal-fetal interface has also been documented both in humans and a variety of animal species (Baker et al., 1993; Hamilton et al., 1998; Han and Carter, 2000; Westwood, 1999). A role for the IGF system in promoting cytotrophoblast migration was suggested by the discovery that the extravillous cytotrophoblast of first trimester express mRNA encoding IGF (Han and Carter, 2000),
together with the demonstration that IGF-1 stimulates trophoblast migration in placental explant culture (Han et al., 1996a; Lacey et al., 2002).

1.2.3. Vascularization of the placenta

The placental and fetal circulations develop independently. A complete feto-placental circulation is established in the sixth week of gestation as the villous trees develop. The development of the villi is accompanied by the establishment of maternal arterial circulation to the placenta. Meanwhile, the concentration of oxygen in the intervillous space increases threefold from 8 to 12 weeks (Jauniaux and Gulbis, 2000). Since the placental tissues, especially the syncytiotrophoblast, contain low levels of principal antioxidant enzymes, the placenta is under oxidative stress at this stage (Myatt and Cui, 2004; Watson et al., 1998). In addition, the circulation dominantly starts at the margin of the chorionic sac and extends to the centre (Jauniaux et al., 2003). Consequently, these factors lead to the discoid shape of placenta.

The onset of maternal arterial circulation and the oxidative stress in the placenta are associated with the pathology of the placenta. Jauniaux et al. describe that the burst of oxidative stress may play a physiological role in stimulating normal placental differentiation, but to some degree, may also be a factor in the pathogenesis of PE and early pregnancy failure if antioxidant defences are depleted (Jauniaux et al., 2000). Autophagy (self-degradation) and apoptosis (programmed cell death) are two crucial, interconnected processes in the placenta that are influenced by oxidative stress. The proper interactions between them play an important role in placental homeostasis. However, an imbalance between the protective and destructive mechanisms of autophagy and apoptosis seems to be linked with pregnancy-related disorders. Premature and disorganized onset of blood flow causes extensive oxidative stress and
villous damage, which may lead to spontaneous miscarriage (Hempstock et al., 2003). Moreover, FGR and PE are also related to increased trophoblast apoptosis and deportation and altered placental vascular reactivity, which result from the inability to mount an effective antioxidant defence against the oxidative stress in the placenta (Burton and Jauniaux, 2004; Myatt and Cui, 2004).

By the end of 18 weeks of gestation, the placenta has attained its definitive form and full thickness and undergoes no further anatomical modifications, though growth in circumference continues until delivery. An elaborate regulatory network is thus established at the feto-maternal interface to maintain a successful pregnancy; however, much remains to be determined before this process is fully understood.

1.3. The mouse as a model of human pregnancy

Animal models have historically played a critical role in the exploration and characterization of disease pathophysiology, target identification, and in the in vivo evaluation of novel therapeutic agents and treatments. In particular, the study of murine placentas has greatly improved our knowledge of placental development and its biology and is useful as a model of human pregnancy due to its small size and short generation time, particularly in the investigation of pregnancy-related pathologies. For example, the endothelial nitric oxide synthase (eNOS) knockout mouse, used as a model of FGR, exhibits impaired uterine artery function and diminished placental System A amino acid transporter activity (Kusinski et al., 2012b). Deletion of the placental-specific Igf-2 gene in mice leads to reduced placental surface area for exchange and increased barrier thickness (Constancia et al., 2002), which is one of the morphological changes in human FGR (Mayhew et al., 2003). Catechol-O-methyl transferase (COMT) is one of the enzymes involved in the metabolism of catecholamine and oestrogen. Deletion of
COMT in pregnant mice leads to human PE-like symptoms (maternal hypertension, increased proteinuria and FGR) (Kanasaki et al., 2008). The heterozygous leptin receptor-deficient mouse develops spontaneous gestational diabetes mellitus (GDM) and the fetuses display insulin resistance similar to infants of human GDM (Yamashita et al., 2003). In addition, human FGR is associated with specific alterations in placental nutrient transporter expression and activity (Cetin, 2003). Mouse models provide an illustration of how placental gene expression, morphology, and nutrient transport can contribute to distinct placental phenotypes (Coan et al., 2010; Gonzalez et al., 2016; Sibley et al., 2005).

Although a number of mouse models of human pathological pregnancies have been established, they inevitably display significant differences from the human disease state, particularly, in terms of placental development and endocrine function.

### 1.3.1. Placental development in mice

Human and rodent placentas are both classified as hemochorial, which means that the chorionic villi are in direct contact with the maternal blood. The placentation of humans and mice shares some structural and functional similarities. Firstly, the behaviour of trophoblast giant cells, which compose of the outer layer of the mouse placenta, is similar to that of extravillous trophoblast cells in humans. Secondly, the function of the labyrinth layer of mouse placenta is mostly analogous to the chorionic villi of human placenta (Georgiades et al., 2002). Additionally, the villi of both human and mouse placentas are covered by syncytiotrophoblast which comes into direct contact with maternal blood (Adamson et al., 2002). However, differences do exist when comparing placental development and endocrine function between humans and mice.
Uterine decidualization is a characteristic of the endometrium of the uterus with implantation of the blastocyst in mammalian species. It involves the transformation of stromal cells in the endometrium from a proliferative to a secretory phenotype. Mice and human differ in the onset and trigger of uterine decidualization. In mice the process of decidualization happens after embryo implantation (Enders and Welsh, 1993), while decidualization begins in the vicinity of the spiral arteries of the endometrium before conception, as early as day 23 of the menstrual cycle in humans (Tang et al., 1994).

In humans the definitive structure of the placenta is established with the vascularization of the chorionic villi as early as day 21 of gestation (Demir et al., 1989). The term ‘definitive placenta’ refers to the stage when nutrient supply to the fetus occurs directly from circulating maternal blood (haemotrophic nutrition) after the feto-maternal circulation is established. The trophoblast invasion is extensive but confined to the inner third of myometrium (Georgiades et al., 2002). Unlike the human placenta, the mouse placenta does not achieve its definitive structure until mid-pregnancy. By day 11.5, the mouse placenta evolves from a choriovitelline pattern to a choriallantoic pattern, which means the chorioallantoic placentas in mice only function for half of the 3 weeks of pregnancy. Further, an extensive fetal capillary network is not seen until day 12.5. From then on, the feto-maternal circulation is established. Trophoblast invasion begins after day 13, which is relatively late compared to humans. Furthermore, trophoblast invasion does not extend to the myometrium, and the invasion is shallow and limited (Adamson et al., 2002). In both humans and mice, NK cells are the dominant leukocytes in the decidua (Felker and Croy, 2016; Hiby et al., 2010). As described in section 1.2.2., human NK and EVT cells have been shown to actively participate in the spiral artery remodelling. However, the uterine artery remodelling in
mice are dependent on NK cells solely, and trophoblast cells are not involved in the process (Adamson et al., 2002).

In the mouse labyrinth, the exchange area of mouse placenta, the branches of the chorionic villi are more interconnected and develop in a maze-like pattern with three layers of trophoblast cells separating the fetal blood from maternal blood. The most remarkable anatomic difference is that mice, in addition to the chorioallantoic placenta, have an inverted yolk sac placenta, which is completely absent from humans; this plays an irreplaceable role in rodent pregnancy with failures leading to embryo malformations (Beckman et al., 1990).

1.3.2. Placental endocrine function in mice

The corpus luteum, a temporary endocrine organ, is essential for establishing and maintaining pregnancy in female mammals. Progesterone, a steroid hormone secreted from the corpus luteum, plays a key role in the decidualization of the endometrium and uterine quiescence (Ozturk and Demir, 2010).

In humans, the corpus luteum develops from an ovarian follicle during the luteal phase of the menstrual cycle. It only lasts for approximately 10 days if the egg is not fertilized. However, if fertilization and implantation occurs, the syncytiotrophoblast cells of the blastocyst begin to secrete human chorionic gonadotropin (hCG) by day 9 after ovulation. hCG ensures progesterone secretion and the maintenance of the corpus luteum during the first 8 weeks of pregnancy. From then on, the corpus luteum decreases progressively and is replaced by the placenta by the end of the first trimester. Apart from hCG and progesterone, other hormones are produced by the syncytiotrophoblast layer of human placenta, including estrogen, placental lactogen and placental growth hormone.
In mice, progesterone is secreted from the corpus luteum throughout pregnancy. During the first 8-9 days of gestation, prolactin from the pituitary gland regulates corpus luteum function. After that time, lactogen secreted by trophoblast giant cells takes over from the pituitary prolactin (Strauss et al., 1996). Placental lactogen is responsible for the maintenance of pregnancy in mice (Brelje et al., 1993).

1.4. Pathological pregnancies

The development of the placenta is essential to successful pregnancy and is a complex but well regulated process. Pathological changes in this process are related to several pregnancy related complications such as FGR, GDM and PE.

1.4.1. Fetal growth restriction

FGR, defined as the fetus that fails to achieve its full genetic growth potential inside the womb, is one of the most prevalent and complex medical complications in modern obstetrics. FGR affects 5–10% of pregnancies worldwide, and is the second cause of infant death during the perinatal period (Bhatia, 2015). In New Zealand, babies of low birth weight (< 2.5 kg) accounted for 5.9-6.2 % of all babies born each year from 2005 to 2014 (Ministry of Health. 2015. Report on Maternity, 2014. Wellington: Ministry of Health). Furthermore, those surviving babies face short term and long term health consequences. It has been shown that FGR is associated with multiple neonatal complications including impaired neurological development, metabolic and hematological disorders (Resnik, 2002). Moreover, FGR infants have an increased risk of disease throughout adulthood, such as obesity, diabetes, hypertension, and stroke (Barker, 2006; Gluckman et al., 2007; McMillen et al., 2001).

Compromised fetal growth is usually defined by a gestational age-specific threshold value of birth weight or ultrasonically estimated fetal weight. The commonly used
threshold value is the 10\textsuperscript{th} percentile (de Onis and Habicht, 1996). Fetal growth can be divided into three categories, according to standardised fetal growth charts: (1) infants with birth weights below 10\textsuperscript{th} percentile, defined as small for gestational age (SGA); (2) infants with birth weights from 10\textsuperscript{th} to 90\textsuperscript{th} percentile, defined as appropriate for gestational age, and (3) infants with birth weights of over 90\textsuperscript{th} percentile, called large for gestational age (LGA). These categories were designed to facilitate further evaluation, rather than diagnosis. SGA was thought to be the proxy for FGR, but they are not synonymous. In fact, SGA infants are not all growth restricted, with only approximately 60\% arising from pathological abnormalities, while others may be constitutionally small or may represent the tail end of a normal distribution (Goldenberg and Cliver, 1997). Additionally, the chart used for fetal growth evaluation is a population-based growth chart and the normal variation in fetal growth potential due to physiological maternal and fetal characteristics (e.g. maternal weight, body mass index, parity, smoking and fetal sex) is not considered, which also leads to misclassification of fetal pathological growth abnormalities (Clausson \textit{et al.}, 2001; Gardosi \textit{et al.}, 1995; Johnsen \textit{et al.}, 2006).

Screening for FGR is a real challenge to all obstetric practitioners, especially in low risk pregnancies. Methods employed for FGR detection include abdominal palpation, symphyseal fundal height measurement, ultrasound biometry, and Doppler flow velocimetry. In addition, a customised fetal growth chart was introduced to reduce false positive and false negative diagnosis of FGR (Gelbaya and Nardo, 2005). Certain biomarkers have also been found to be associated with FGR and may predict the occurrence and the outcome of FGR (Franco \textit{et al.}, 2007; Llanos and Ronco, 2009).

The aetiology of FGR is multifactorial and involves maternal, fetal and placental factors (Table 1.1). On the maternal side, hypertension disorders, especially PE, are
associated with a 4-fold increase in the risk of FGR (Kovo et al., 2015). In addition, maternal diabetes and obesity may also lead to restricted fetal growth, although their effects may be paradoxical (Langer et al., 1989; Morrison et al., 2010; Scholl et al., 2001). A cohort study in the UK showed that maternal obesity was associated with aberrant fetal growth, encompassing both growth restricted and macrosomic fetuses (Higgins et al., 2011). Similar effects were also observed in animals. Jones et al. fed mice a high fat diet for 8 weeks before mating and throughout gestation, resulting in fetal overgrowth (Jones et al., 2009), whereas Jungheim et al. observed smaller fetuses in mice fed with high fat diet for 16 weeks (Jungheim et al., 2010). On the fetal side, aneuploidy, fetal malformations, congenital infections and multiple gestations contribute to FGR (Bahado-Singh et al., 1997; Khoury et al., 1988). Of all the factors, “placental insufficiency” is believed to be one of the dominant contributors (Maulik, 2006). Placental insufficiency describes an inability of the placenta to supply sufficient oxygen and nutrients to support adequate fetal growth, and may be due to inappropriate maternal/fetal blood flow, reduced nutrient transfer and/or morphological abnormalities of the placenta. Firstly, adequate blood supply is essential for a normally functioning placenta. FGR fetuses exhibit persistent reductions in umbilical vein blood flow (Rigano et al., 2001) and abnormal uterine artery waveforms are associated with FGR (positive likelihood ratio 14.6) (Cnossen et al., 2008). The sensitivity of the uterine artery velocity waveforms for the detection of FGR is 11.7% (Martin et al., 2001). Secondly, in addition to adequate blood supply, the ability of placenta to transfer nutrients from the mother to the fetus is crucial to fetal development. Previous studies have observed that a reduction in nutrient transport across placenta is associated with FGR (Glazier et al., 1997; Jansson et al., 2002; Norberg et al., 1998). Jansson et al. also demonstrated that placental amino acid transport is down-regulated prior to the
development of FGR (Jansson et al., 2006). It has been suggested that the placenta may function as a nutrient sensor, as placental transport alterations represent a mechanism to match fetal growth rate to a level which is compatible with the amount of nutrients that can be provided by the maternal supply (Jansson and Powell, 2006). Thirdly, FGR pregnancies are always accompanied by morphological abnormalities of the placenta (Mifsud and Sebire, 2014). Abnormal development, inadequate perfusion, and dysfunction of the placental villi contribute to the development of FGR. Diminished trophoblast invasion compromises the growth and development of the fetal vasculature and ultimately the growth of the placental villi, resulting in reduced blood flow in FGR pregnancies (Egbor et al., 2006). Macara et al. found that terminal villi from FGR cases were smaller in diameter and had several abnormal features in comparison with controls (Macara et al., 1996). Studies compared morphometric measures of villous development in normal pregnancies with those complicated by FGR and found that FGR was associated with abnormal growth of villi and fetal capillaries (Mayhew et al., 2003; Mayhew et al., 2004).
Table 1.1: The aetiology of fetal growth restriction (Bernstein and Divon, 1997)

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<td>Aneuploidy (trisomy 13, 18 and 21, triploidy)</td>
<td>Placenta previa</td>
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<td>Renal disease</td>
<td>Fetal malformations (gastrochisis, omphalocele, congenital heart defect)</td>
<td>Abruptio placentae</td>
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<td>Diabetes</td>
<td>Multiple gestation</td>
<td>Infarction</td>
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<td>Vascular disease</td>
<td>Infection (toxoplasmosis, rubella, cytomegalovirus, herpes)</td>
<td>Circumvallate placenta</td>
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<td>Autoimmune disorders (systemic lupus erythematosus, antiphospholipid)</td>
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<td>Placenta accreta</td>
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<td>Hypoxia (pulmonary disease, cyanotic cardiac disease)</td>
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A number of biomarkers for predicting FGR have been proposed, including biomarkers related to angiogenesis, endothelial function and placental hormones. However, none of these biomarkers are sufficiently accurate to recommend their use in clinical practice (Conde-Agudelo et al., 2013). Currently there are no definite and curative antenatal treatments for FGR. Once FGR is established, assessment of fetal well-being and timely delivery remain the main management strategy (Figuera and Gratacos, 2014). Certain drugs, such as aspirin, arginine, heparin, and sildenafil (Riyazi et al., 1998; Satterfield et al., 2010; Uzan et al., 1991; Xiao and Li, 2005), as well as some growth factors, such as GH-N and IGF-1 (Eremia et al., 2007; Roberts et al., 2008; Tung et al., 2012), have been introduced as potential treatments for FGR, but their effectiveness still needs further evaluation.
1.4.2. Gestational diabetes mellitus

GDM is a condition in which women without previously diagnosed diabetes exhibit high blood glucose during pregnancy (American Diabetes, 2014). It is associated with multiple gestational complications, including macrosomia, dystocia, stillbirth, hypoglycaemia and respiratory distress (Langer et al., 2005). Both the fetus and the mother have increased risk for diabetes in later life (Catalano et al., 2003). The aetiology of GDM is unclear. Normal pregnancy presents insulin resistance that begins mid-pregnancy and progresses through the third trimester to levels that approximate the insulin resistance seen in type 2 diabetes patients (Barbour et al., 2007). This insulin resistance appears to result from a combination of increased maternal adiposity and the anti-insulin effects of hormonal products of the placenta (Ryan and Enns, 1988). The fact that insulin resistance rapidly abates following delivery also suggests that the major contributors to this state of resistance are placental hormones.

1.4.3. Pre-eclampsia

PE is a complication of pregnancy, defined as new-onset, persistent hypertension with either proteinuria or multisystem disease occurring at 20 weeks’ gestation or later (Brown et al., 2001). PE is one of the leading causes of maternal, fetal, and neonatal mortality and morbidity. It affects 3–5% of pregnancies worldwide and is characterized by maternal hypertension, proteinuria, increased vascular injury and permeability, and shallow placental invasion, and if left untreated can progress to maternal multiorgan failure, coagulopathy and seizures (Friedman et al., 1991b). PE can be characterized into 2 different disease entities: early-onset PE and late-onset PE (von Dadelszen et al., 2003). Early-onset PE is usually defined as PE that develops before 34 weeks of gestation, whereas late-onset PE develops at or after 34 weeks of gestation. Although they present some overlapping features, different maternal and fetal outcomes,
biochemical markers, heritability, and clinical features are associated with them respectively (Raymond and Peterson, 2011).

The aetiology of PE is not fully understood. Two stages of the PE syndrome have been proposed: (1) poor trophoblast invasion, and (2) a systemic, maternal-inflammatory response. Defects in the invasion of the decidua by the trophoblasts during the implantation period, lead to impaired remodelling of the spiral arteries of the decidua and myometrium during the first and second trimesters. A leading theory of the cause of this defect is an exaggerated maternal immune response to the trophoblasts, which may be related to maternal genetic factors (Steegers et al., 2010). Failed remodelling may result in high resistance blood flow. This is the first stage of PE, which occurs prior to the development of any clinical signs or symptoms. The second stage of PE occurs during the second half of pregnancy and is associated with an exaggerated endothelial activation and a generalised hyperinflammatory state. As the growing fetus puts additional demands on the placenta, it struggles with its inadequate or intermittently inadequate blood flow. The resulting hypoxia and oxidative stress lead to release of excessive amounts of inflammatory cytokines, microparticles and anti-angiogenic factors (Sargent et al., 2006). Altogether, this leads to the generalized inflammation and endothelial dysfunction and associated increased vascular reactivity that produce the clinical signs and symptoms of PE. Recent studies have suggested that the anti-angiogenic state in PE is mediated by high circulating levels of soluble Fms-like tyrosine kinase 1 (sFlt1) and soluble endoglin in concert with low levels of pro-angiogenic factors (Alasztics et al., 2014; Fan et al., 2014; Maynard et al., 2003). sFlt-1 antagonizes and reduces bioavailable vascular endothelial growth factor (VEGF) (Palmer et al., 2015) and is elevated in reduced uterine perfusion pressure rat model of PE (Ushida et al., 2016). Epidemiologic studies have revealed altered circulating levels
of anti-angiogenic proteins (sFlt1, endoglin) in women with PE both during and prior to clinical signs and symptoms of the disease, consistent with a pathogenic role for these anti-angiogenic factors in PE (Levine et al., 2006; Noori et al., 2010; Romero et al., 2008).

Once the diagnosis of PE is made, the only known definitive treatment for PE is delivery of the fetus and placenta. Management consists of close observation of the maternal and fetal condition, control of hypertension, prevention and treatment of seizures and timely delivery before maternal or fetal compromise occurs. Currently, there are no treatments in clinical practice that stabilize or reverse the pathology of PE.

Current knowledge of the pathophysiology of PE has identified several promising targets which may provide an opportunity for the development of therapies for this disease. These include restoring the balance of pro-angiogenic and anti-angiogenic factors, the balance of vasodilation and vasoconstriction, reducing inflammation and oxidative stress (Oyston et al., 2015). Although clinical studies are already underway, conclusive evidence of benefit and safety of those treatments are still likely to be years away.

1.5. Growth hormone and pregnancy

1.5.1. Physiology of growth hormone

GH is a classical endocrine hormone secreted mainly by the somatotropic cells in the anterior pituitary which has widespread effects on multiple tissues in the body, including increasing the mineralization of bone and muscle mass, promoting lipolysis and gluconeogenesis in the liver, and stimulating homeostasis and immune system (Table 1.2). Among these, the somatic growth stimulating effect of GH has been debated for over half a century. The original somatomedin hypothesis was coined in
1950’s. Animal experiments showed that GH did not act directly on its target tissue and a hypothesis was put forward that the effect of GH was mediated by a substance termed “somatomedin”. Somatic growth was regulated by GH’s stimulation of hepatic somatomedin, known as IGF, with IGFs acting as endocrine factors to promote growth. However, GH has been found to have additional IGF-independent effect on growth (Wu et al., 2015). Apart from this, GH is also secreted at a number of extrapituitary sites, including the brain, immune system (including the thymus, spleen, tonsils, lymph nodes, and lymphocytes), mammary gland, testis and placenta, where it has localised autocrine/paracrine effects (Harvey and Hull, 1997; Harvey et al., 2000b; Harvey et al., 1998), adding to the complex functions of GH.

Secretion of GH from the pituitary is controlled by neuronal, hormonal and metabolic factors. The hypothalamus releases GH-releasing hormone (GHRH) which increases GH secretion from the somatotroph cells of the anterior pituitary, and somatostatin, also known as GH-inhibiting hormone (GHIH), which inhibits GH secretion. GHRH stimulates GH synthesis as well as secretion, while somatostatin blocks only secretion and is therefore thought to be important to allow GH accumulation in somatotrophs ready for release when next stimulated (Thorner et al., 1990). A characteristic feature of GH secretion is the pulsatile pattern with several peaks each day (Bluet-Pajot et al., 1998). These peaks are primarily affected by the onset of deep sleep, food intake and exercise. The GH axis also demonstrates sex-specific dimorphic release patterns. Men have large nocturnal GH pulses while women have more continuous GH secretion and more frequent GH pulses during a day (Jaffe et al., 1998). Besides, a number of factors seem to affect GH secretion in different aspects (Table 1.3).
Table 1.2: Principal biological activities of GH (Isaksson et al., 1985)

- Promotion of somatic growth
- Calcium and sodium retention
- Amino acid transport
- Lipolysis
- Protein synthesis
- Homeostasis
- Insulin antagonism
- Beta cell hyperplasia
- Lactogenesis
- Stimulation of the immune system

Table 1.3: Stimulators and inhibitors of GH secretion (Frohman et al., 1992)

<table>
<thead>
<tr>
<th>Stimulators</th>
<th>Inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHRH</td>
<td>Somatostatin</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>GH and IGF-1 negative feedback</td>
</tr>
<tr>
<td>Androgen and estrogen</td>
<td>Hyperglycaemia</td>
</tr>
<tr>
<td>Clonidine</td>
<td>Glucocorticoids</td>
</tr>
<tr>
<td>Hypoglycaemia</td>
<td>Dihydrotestosterone</td>
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<tr>
<td>Arginine</td>
<td></td>
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<tr>
<td>Niacin</td>
<td></td>
</tr>
<tr>
<td>Deep sleep</td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td></td>
</tr>
</tbody>
</table>

1.5.2. The growth hormone gene cluster

The human GH gene family is a cluster of five tandemly arranged and highly related genes, which includes *GH-N* (*GH1*), *GH-V* (*GH2*), and three chorionic somatomammotropins (*CS*; also known as placental lactogen, *PL*) *CS-A*, *CS-B* and *CS-L* (Fiddes et al., 1979). Each of these genes is composed of five exons (1 to 5) and four introns (A to D) occurring at identical positions (Seeburg, 1982). They have evolved from the same ancestral precursor located in a 47-kb cluster on the long arm of chromosome 17 (q22-q24) (George et al., 1981). The order of these genes is *GH-N*, *CS-L*, *CS-A*, *GH-V* and *CS-B*, with a locus control region (LCR) located upstream of the GH-N gene (Jones et al., 1995) (Figure 1.2). This locus control region is comprised of 5
DNase I hypersensitive sites within the *GH-N* promoter (Jones et al., 1995) and controls tissue specific expression of the GH locus (Su et al., 2000). *GH-N* is expressed primarily in pituitary somatotroph cells and at certain extrapituitary sites, as described above. The remaining four genes are expressed in the syncytiotrophoblast cells of the placenta. Distinct patterns of chromatin modification are associated with activation of the human GH cluster in the pituitary and the placenta (Kimura et al., 2004).

Figure 1.2: The human GH gene cluster. The human GH gene family is a cluster of five genes, including *GH-N*, *CS-L*, *CS-A*, *GH-V* and *CS-B*. *GH-N* is expressed primarily in the pituitary. The remaining four genes are expressed in the placenta.

The members of the GH gene family share 91% to 99% sequence nucleotide identity in the coding regions, and are consequently thought to have arisen by gene duplication (Barsh et al., 1983). The *GH-N* gene codes for the pituitary GH protein, and the *CS-A* and *CS-B* genes are spliced and encode identical CSs. The *GH-V* gene was originally thought to be a pseudogene until expression of *GH-V* mRNA was identified in the human placenta in 1987 (Frankenne et al., 1987). The expression of *GH-V* has been demonstrated to be tightly coupled to placental differentiation (Carney et al., 1993). Whether the *CS-L* gene is translated into a protein is still unclear.

In contrast to the situation in the human genomes, mouse *Gh* is encoded by only a single gene located in chromosome 11 (Das et al., 1996). A redundant prolactin gene
family has been detected in mice, whereas only a single prolactin gene, expressed in the pituitary, exists in humans (Wiemers et al., 2003). The differences between the structure of the human and mouse \(Gh\) genes may have important implications for the respective modes of gene regulation in these two species.

1.5.3. **Growth hormone isoforms**

To date, a number of GH isoforms have been identified. This heterogenetic phenomenon may be due to several reasons. At the genetic level, two different genes encode GH: GH-N gene and GH-V gene. Due to alternative splicing, different mRNAs can be produced from these genes. At the mRNA level, post-translational processing can also contribute to the GH isoform family.

There are two main isoforms of pituitary GH-N, produced by alternative splicing: a 191 amino acid protein, 22 kDa GH-N, which is the prototype, and a second 176 amino acid isoform, 20 kDa GH-N. A third isoform of GH-N was discovered in 1987, a 17.5 kDa variant produced by alternative splicing, although it was originally thought to be only expressed under pathological conditions (Lewis, 1992). The GH-V gene also has at least four product proteins: a 22 kDa GH-V, a 25 kDa glycosylated GH-V, a 20 kDa GH-V and a 24 kDa GH-V (Frankenne et al., 1990). The dominant product of the GH-V gene, 22 kDa GH-V, is a single chain protein with an N-glycosylation site at position 140–142. It differs from 22 kDa GH-N by 13 amino acids and has a higher isoelectric point and weaker lactogenic properties (Igout et al., 1995).

The 22 kDa GH-N is the most abundant and major bioactive form of GH, comprising 85-90% of circulating GH-N isoforms, while the 20 kDa GH-N accounts for approximately 10% of the pituitary GH-N transcripts. Both 20 kDa and 22 kDa GH-N bind with similar affinity to the GH receptor (GHR) and have equipotent effect in growth promotion, lipolytic activity and body fat reduction (Isaksson et al., 1987;
Takahashi and Satozawa, 2002). However, some studies have observed that 20 kDa GH-N has weaker antidiuretic and diminished diabetogenic actions (Takahashi et al., 2001). In addition, both 22 kDa and 20 kDa GH-N can bind with and activate the prolactin receptor, but 20 kDa GH-N interacts poorly, resulting in weak lactogenic effects (Wada et al., 1998).

Similarly, 22 kDa GH-V is the main circulating form of GH-V, while the expression of the 20 kDa GH-V seems to be variable in normal and pathological conditions. 20 kDa GH-V is generated from a 45-bp deletion produced by the use of an alternative splice acceptor site within exon 3, similar to that in the GH-N gene. However, the transcript encoding 20 kDa GH-V is not detected in all placentas, which may partly explain the previous unsuccessfully attempts in detecting this transcript (Boguszewski et al., 1998).

In an animal study using 22 kDa GH-N and 20 kDa GH-V, Vickers et al. observed that 20 kDa GH-V shared some of the growth-promoting and all antilipogenic activities of pituitary 22 kDa GH-N but had reduced lactogenic and diabetogenic properties (Vickers et al., 2009).

In addition to the key isoforms for each protein described, there may be other minor isoforms generated by alternative splicing. Subjected to different processes of pre- or post-translation of GH gene, such as mRNA splicing, acetylation, deamidation, and oligomerization, the GH isoform family may comprise over 100 variants.

1.5.4. The growth hormone receptor

As a protein hormone, GH mediates anabolic effects on the body by interacting with the specific receptors on the plasma membrane of target cells. The GHR is a member of the Type I cytokine receptor family and has three domains characteristic of this family: an
extracellular binding domain, a transmembrane domain and an intracellular domain for signalling (Bazan, 1990).

The human GHR gene is located on the short arm of chromosome 5 (5q13-q12) and has 9 exons (exons 2-10) (Godowski et al., 1989). Exon 2 codes for the signal peptide, exons 3-7 code for the extracellular domain, exon 8 encodes the transmembrane domain, and exons 9 and 10 encode the cytoplasmic domain (Amselem et al., 1989). The mouse GHR gene is composed of 11 coding exons. Nine of the coding exons are homologous in size and sequence to the 9 coding exons of the human GHR gene. The other two coding exons have been designated exons 8A and 4B. Exon 8A encodes the hydrophilic tail of mouse GHR, while exon 4B encodes an 8-amino acid segment of the ligand binding domain that is unique to mouse GHR (Moffat et al., 1999).

There are at least two GHR isoforms which have been identified, a full-length isoform (fl-GHR) and a shorter one which lacks exon 3 (d3-GHR). The latter was discovered by Frankenne et al. in the trophoblast cells of the placenta in 1992 (Frankenne et al., 1992). These two isoforms differ by the retention or exclusion of exon 3 during splicing. The allele encoding d3-GHR exists in humans only (Pantel et al., 2003). Humans have three GHR genotypes: fl/fl, d3/fl and d3/d3. The relationship between the GHR genotype and somatic growth promoting effect of GH is still controversial. Although both GHR isoforms have been found to have a similar affinity for GH (Sobrier et al., 1993), there are still some studies that have observed that GHR polymorphisms may influence the effects of GH. Dos Santos et al. found that patients with d3-GHR expression were associated with 1.7 to 2.0-times higher growth acceleration than patients with only fl-GHR expression in two cohorts of short children treated with GH (Dos Santos et al., 2004). Binder et al. also found that the patients with d3-GHR genotype had increased responsiveness to GH (Binder et al., 2006). However,
a two-year controlled prospective study in 170 Spanish patients did not observe any difference between the GHR genotypes in responsiveness to GH treatment (Carrascosa et al., 2006). In a prospective study of 142 short prepubertal children born SGA, the authors also suggested that the d3-GHR allele could only predict better GH responders in the first year treatment (Dorr et al., 2011). Each of the studies mentioned above relate to the role of d3-GHR in postnatal growth and the responsiveness to GH treatment. A study was also carried out by Padidela et al. on d3-GHR in antenatal growth (Padidela et al., 2012). This study analysed the GHR genotype in a cohort study in the UK and found that fetal GHR genotype was associated with placental and birth weight but not related to antenatal anthropometric measurements or growth in infancy.

In 1992, De Vos et al. determined that GH interacted with the extracellular domain of its receptor though determining the crystal structure complex (de Vos et al., 1992). GH possesses two receptor binding sites: Site 1, a high affinity site; and Site 2, a lower affinity site. One GH molecule interacts with the extracellular domains of two receptors, and this process is sequential. The initial step is the binding of the high affinity site of GH to GHR. Then the lower affinity site contacts the second GHR to stabilize the GH receptor complex (Cunningham et al., 1991). After the formation of the dimerization, the signal transduction pathway is activated (Wells, 1996). However, the GHR actually exists as a constitute dimer consisting of two GHR subunits (Brooks et al., 2008). Recently, Brooks et al. further demonstrated the mechanism of activation of the GHR by GH (Brooks et al., 2014) (Figure 1.3).
Figure 1.3: The structure of GHR and the process of janus kinase (JAK) 2 activation. A: JAK2 consists of a FERM domain, a pseudokinase domain and a tyrosine kinase domain. JAK2 binds with the cytoplasmic domain of GHR via its FERM domain (Ghoreschi et al., 2009). In the GHR unliganded state, the pseudokinase domain interacts with the tyrosine kinase domain and inhibits its activity (Waters et al., 2006). B: After GH is captured and binds with two extracellular domains via Site 1 and Site 2, transmembrane domains of the receptor are drawn and converted to a left-hand crossover state from the parallel from, resulting in the separation of the cytoplasmic domains. This separation leads to the removal of the pseudokinase domains and the contact of the tyrosine kinase domains, triggering JAK2 activation.

1.5.5. Growth hormone receptor-mediated signal transduction

The GHR lacks intrinsic tyrosine kinase activity, and therefore relies on the recruitment of additional signalling molecules to mediate signal transduction. Key pathways include the janus kinase-signal transducer and activator of transcription (JAK-STAT), mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase (PI3-K) pathways (Figure 1.4). Both JAK-STAT and MAPK pathways lead to transcriptional activation, while the activation of the PI3-K results in the induction of the insulin-like action of GH (Herrington and Carter-Su, 2001).
The JAK-STAT pathway: The investigation of the cytokine receptor superfamily led to the discovery of the JAK-STAT signalling pathway. It consists of three key components: a receptor in combination with members from the JAK and STAT family. Despite the lack of intrinsic tyrosine kinase activity of the GHR, activation induces tyrosyl phosphorylation of the intracellular domain by activating non-receptor tyrosine kinases from the JAK family (Schindler and Darnell, 1995). JAK plays an important role in the initial steps of the JAK-STAT pathway signalling and it consists of four members: JAK1, JAK2, JAK3 and tyrosine kinase 2. JAK2 is the predominant member of the family associated with the GHR, but an association with JAK1 has also been described in some tissues in mice (Hellgren et al., 2001). JAK2 was first identified as a GH receptor-associated tyrosine kinase in 1993 (Argetsinger et al., 1993). The binding of GH to the GHR triggers activation of JAK2, leading to autophosphorylation of the kinase and phosphorylation of the intracellular domain of the GHR. Subsequently, several intracellular substrates including the STAT family are phosphorylated (Carter-Su et al., 1996). STAT proteins are latent cytoplasmic transcription factors that require phosphorylation for nuclear retention. Once STAT proteins are activated, they bind to a consensus DNA-recognition motif called gamma-activated sites and activate transcription of the cytokine-inducible genes (Ihle, 1996) (Figure 1.3). Seven mammalian STAT proteins have been identified: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6. Among these, STAT1, STAT3 and STAT5 have been found to be important to the GH signalling pathway (Ram et al., 1996). The STATs have specific target genes. For example, STAT1 and STAT3 bind to the sis-inducible element as a regulatory element in the GH activation of c-fos gene transcription. In addition, studies in mice have showed that serum IGF-1 levels are reduced in STAT5-deficient mice, indicating that the Igf-1 gene might be a direct target of STAT5 (Cui et
al., 2004; Engblom et al., 2007). The genes encoding STAT5A and STAT5B are highly homologous and share 96% identity in the coding region (Kisseleva et al., 2002). Consequently, STAT5A and STAT5B play both overlapping and distinct roles in GH signalling (Liu et al., 1995). However, animal studies have demonstrated that STAT5A and STAT5B single knockout mice have remarkably distinct phenotypes (Liu et al., 1997; Teglund et al., 1998). Additionally, STAT5B knockout mice exhibited defects similar to the GHR deficient mice (Udy et al., 1997), thus STAT5B may be a key agent linking GH stimulated signals and IGF-1 gene expression (Chia et al., 2010). Silva and Kloth et al. also observed that both GH-N and GH-V share the same signal transduction pathway by activating STAT5B in GHR expressing cell models (Silva et al., 2002).

**MAPK pathway:** The initial step of the MAPK pathway is the phosphorylation of SHC after the autophosphorylation of JAK2, followed by a sequential cascade including Grb2, son-of-sevenless (SOS), Ras, Raf and MAP/ERK kinase (MEK) (English et al., 1999).

**PI3-K pathway:** In addition to activating the MAPK pathway, GH has also been shown to stimulate PI3-K pathway. GH has long been known to have both insulin-like and anti-insulin-like effects on carbohydrate and lipid metabolism (Davidson, 1987), suggesting that GHR and insulin receptor may share some signalling pathways. Consistent with this, GH stimulates phosphorylation of the insulin receptor substrates (IRS) after the activation of JAK2 (Carter-Su et al., 1996). Similar to the action of insulin, GH induces the tyrosyl phosphorylation of IRS proteins, providing binding sites for the regulatory subunits of PI3-K (Ridderstrale et al., 1995; Sun et al., 1991). GH activation of PI3-K via IRS proteins plays an important role in glucose transport and lipid synthesis (Takano et al., 2001).
Figure 1.4: GHR-mediated signal transduction pathways. Activation of the GHR activates 3 key signal pathways: JAK-STAT, MAPK and PI3-K pathways. Both JAK-STAT and MAPK pathways lead to transcriptional activation, while the activation of the PI3-K results in the induction of the insulin-like action of GH.

1.5.6. Pituitary growth hormone and placental growth hormone

Although GH-N and GH-V stem from the same gene cluster and their proteins only differ by 13 amino acids, they are quite different in certain aspects. GH-N is secreted in the anterior pituitary by somatotropic cells in a pulsatile manner. The secretion rate of pituitary GH changes rapidly, regulated by a series of positive and negative stimuli. However, GH-V is specifically expressed in the syncytiotrophoblast layer of the human placenta and secretion is continuous, which has important implications for physiological adjustment to gestation. Similar to GH-N, the secretion of GH-V is inhibited by hyperglycaemia (Patel et al., 1995). However, GH-V is not regulated by GHRH, ghrelin or somatostatin. The secretion and the maternal level of GH-V is closely related to the formation of the syncytiotrophoblast (Alsat et al., 1998). GH-V
levels are also affected by fetal gender (Chellakooty et al., 2002). Recent studies have observed that maternal body mass index (BMI) is negatively correlated to the level of GH-V during different stages of pregnancy, and leptin may play a key role in this phenomenon (Coutant et al., 2001; Verhaeghe et al., 2002).

GH-N and GH-V bind the GHR with similar affinity and share similar physiological effects on somatotrophic, lactogenic and lipolytic properties, as well as the effect on immunoregulatory process (Goodman et al., 1991; Hansen, 2003). However, GH-V binds the prolactin receptor poorly and its lactogenic affects are greatly reduced compared with GH-N (Verhaeghe, 2008). However, the physiology of GH-V is far from understood.

1.5.7. Placental growth hormone in normal pregnancy

GH-V is thought to play a key role in the maternal adaptation and fetal growth (Caufriez et al., 1993). Firstly, it is thought that GH-V stimulates growth and maturation via IGF-1 during pregnancy. Secondly, GH-V induces maternal insulin resistance to ensure supply of nutrients is adequate for the growing fetus (Barbour et al., 2002). In addition, GH-V influences the process of placentation (Handwerger and Freemark, 2000).

In human pregnancy, pulsatile pituitary-derived GH is the main form of GH in maternal circulation before 15 weeks of gestation (Eriksson et al., 1988). Following placentation, GH-V is detected from as early as 5 week gestation and levels increase significantly to reach peak levels at approximately 37 week gestation (Chellakooty et al., 2004). A positive association between serum levels of GH-V and IGF-1 has been observed in some studies (Caufriez et al., 1990a; Caufriez et al., 1994). At approximately 17 weeks gestation, GH-V replaces GH-N completely, resulting from the negative feedback by
GH-V and IGF-1 (Eriksson et al., 1989). With the onset of labour, there is a rapid fall of GH-V levels in maternal serum, which occurs within 1 h after birth, contributed to by the 15 min half-life of GH-V in the circulation and placental origin of the hormone (Lonberg et al., 2003).

Numerous studies have demonstrated that GH-V is positively related to fetal growth (Chellakooty et al., 2004; Koutsaki et al., 2011; McIntyre et al., 2000; Sifakis et al., 2012b). In addition, a positive correlation between GH-V and IGF-1 has also been observed in both longitudinal and cross-sectional studies (Caufriez et al., 1990a; Caufriez et al., 1993) and it is thought that GH-V regulates the circulating IGF-1 values during pregnancy (Handwerger and Freemark, 2000).

Prior investigations suggested that GH-V is only secreted into the maternal circulation as GH-V had not been detected in fetal blood. It was therefore thought that GH-V impacted on fetal growth by regulating the maternal substrate supply via IGF-1 (Caufriez et al., 1990a). However, conflicting results exist on the relationship between maternal IGF-1 level and fetal growth during pregnancy (Caufriez et al., 1994; Chellakooty et al., 2004). As maternal IGF-1 level is not closely related to fetal growth, GH-V may influence fetal growth through alternative mechanisms. In support of this, the presence of GH receptor in the placenta and the stimulation of trophoblast invasion by GH-V have been observed in several studies (Frankenne et al., 1992; Hill et al., 1992; Lacroix et al., 2005; Mertani et al., 1995). Thus, it is argued that GH-V may play a role in placental function and the process of placentation, but the mechanism remains to be defined.

Recently, GH-V was detected in umbilical cord samples using a highly sensitive enzyme-linked immunosorbent chemiluminiscent assay in a cross-sectional study.
(Mittal et al., 2007a). This is the first evidence that GH-V exists in the fetal circulation, and is contrary to the popular belief that GH-V is secreted by the placenta only into the maternal circulation. Another study by Higgins et al. in 2012 also observed the presence of GH-V in fetal circulation and at levels similar to the previous report (Higgins et al., 2012). It is thought that only substances under 1 kDa can cross the placental barrier (Illsley, 2000). Therefore, GH-V may be secreted directly from the syncytiotrophoblast into the fetal circulation rather than cross the placental from maternal circulation, or it may be actively transported across. However, fetal GH-V levels are much lower than maternal levels, and do not appear to be related to fetal growth and placental size (Higgins et al., 2012). Given the above findings, the current understanding of GH-V function in the fetus needs to be re-evaluated.

1.5.8. Regulation of placental growth hormone

The regulation of GH-V is still unclear. Several stimulators and inhibitors of pituitary GH secretion, including GHRH, ghrelin and somatostatin, have been shown to have no effect on GH-V levels (Caron et al., 1997; de Zegher et al., 1990; Fuglsang et al., 2005b). However, some studies found GH-V secretion may be related to maternal glucose level. Glucose inhibits GH-V secretion in vitro. In vivo, hypoglycaemia was induced in insulin-dependent diabetes pregnancies and a marked increase on maternal circulating GH-V concentrations was observed (Bjorklund et al., 1998; Patel et al., 1995). Additionally, the adipokine leptin is expressed in placenta and fetal tissues (Harris, 2000) and was thought to have physiological effects on the placenta and its function given the marked state of leptin resistance during pregnancy (Grattan et al., 2007; Hoggard et al., 2001). Indeed, some studies found that maternal leptin acts as a negative predictor of GH-V (Coutant et al., 2001; Verhaeghe et al., 2002), but the mechanisms are not well defined.
1.5.9. Placental growth hormone in pathological pregnancies

GH-V is associated with a number of pathological pregnancy conditions, including FGR, GDM and PE.

**Fetal growth restriction**

As described in Section 1.4.1., the aetiology of FGR is multifactorial and involves maternal, fetal and placental factors. Of all the factors, “placental insufficiency” is believed to be one of the dominant contributors (Maulik, 2006). Placental insufficiency includes inappropriate maternal/fetal blood flow, reduced nutrient transfer and morphological abnormalities of the placenta (Kusinski *et al.*, 2012a). Several studies have demonstrated a positive relationship between maternal GH-V serum concentrations and fetal growth. Additionally, they demonstrate lower concentrations of GH-V in pregnancies complicated by FGR. Veronique Mirlesse *et al.* found that lower concentrations of GH-V in maternal plasma samples taken after 31 weeks until term in 22 cases of FGR (Mirlesse *et al.*, 1993). In a study conducted by McIntyre *et al.*, blood samples were obtained from FGR pregnancies (n=16) at 28-30 weeks gestation and 36-38 weeks gestation, and lower concentrations of GH-V was also observed at both time points (McIntyre *et al.*, 2000). Further, Mannik *et al.* described the expression of GH-V in the placenta from SGA pregnancies (Mannik *et al.*, 2010). Placental samples were collected from 72 pregnancies after cesarean section or vaginal delivery and GH-V expression was determined via reverse transcription polymerase chain reaction (RT-PCR). Compared with normal-size babies, the expression of GH-V was about 1.1 fold lower in SGA. On the other hand, it has been shown that there are no significant association between maternal serum GH-V and birth weight in early pregnancy (11-13 weeks) (Sifakis *et al.*, 2012c). Low GH-V in FGR/SGA pregnancies in late pregnancy
may therefore reflect the aetiology of FGR – i.e. a poorly developed placenta synthesising less GH-V.

As described in Section 1.1., the placenta receives blood supply from both the maternal and the fetus, and thus has two separate circulations: the uteroplacental circulation and the fetoplacental circulation. The maternal blood flow is supplied by the uterine and ovarian arteries while the fetal blood flow is derived from the umbilical arteries. Between these two circulatory systems, exchange of oxygen and nutrients take place in the intervillous space in the placenta. Any impairment among the maternal and fetal blood flow and the placenta can lead to reduce blood supply to the fetus, resulting to fetal growth restriction. Schiessl et al. observed impaired uterine blood flow is correlated with low level serum level of placental in FGR pregnancies and indicated that lower level of GH-V might contribute to the impaired uteroplacental circulation (Schiessl et al., 2007). This may be mediated through secondary regulation of IGF-1, as IGF-1 has been demonstrated to directly alter human myometrial arterial tone using wire myography (Corcoran et al., 2012). However, whether GH-V has similar impacts on the placental or myometrial arteries is not known.

As mentioned above, the placenta plays an important role between maternal and fetal circulation. The rates of placental blood flow are dependent on placental vascularization and angiogenesis. Although a number of factors have been implicated in angiogenesis, fibroblast growth factor (FGF) and VEGF are key angiogenic factors during placental angiogenesis (Reynolds and Redmer, 2001). The failure of vascularization and angiogenesis lead to increased vascular resistance and reduced blood flow, and are associated with high-risk pregnancies including PE and FGR (Harrington et al., 1997a; North et al., 1994). The proteins in the prolactin (PRL) and GH family and derived peptides have been revealed as the potent regulators of
angiogenesis and control the initiation and then the cessation of placental vascularization (Jackson et al., 1994). Further, In vivo and in vitro studies showed that this opposing action is mediated by the intact and N-terminal fragments of PRL/GH family (Ferrara et al., 1991; Gould et al., 1995). The 16 kDa N-terminal fragments cleaved form PRL, pituitary GH and GH-V are antiangiogenic while the intact PRL, pituitary GH and GH-V proteins are angiogenic, and their effects present via the activation of the MAPK signalling pathway (Mudgett et al., 2000). The 16 kDa N-terminal fragments inhibit the activation and tyrosine phosphorylation of MAPKs, resulting in the downstream of the phosphorylation of FGF and VEGF receptor (D'Angelo et al., 1999; Struman et al., 1999). On the other hand, placental vascularization begins with the invasion of trophoblast into the uterus. Deficient trophoblast invasion of the placental bed spiral arteries is another crucial factor to the pathogenesis of FGR (Kaufmann et al., 2003). The precise mechanisms that regulate trophoblast invasion are largely unknown with several proteinases, cytokines, and growth factors involved. Lacroix et al. determined that both GH-V and pituitary GH can stimulate trophoblast invasion, but GH-V was more efficient in stimulating invasiveness (Lacroix et al., 2005). This result implicates an autocrine or paracrine role of GH-V in the regulation of trophoblast invasion.

Apart from blood supply, placental nutrient transport capacity is another major contributor of FGR. However, all substrates that pass between the maternal and fetal circulation must go through the placental exchange barrier which consists of a number of layers: syncytiotrophoblast, discontinuous inner cytotrophoblast layer, basal lamina of the trophoblast, connective (mesenchymal) tissue of the villus, basal lamina of the endothelium and endothelium of the fetal placental capillary in the tertiary villus (Leiser and Kaufmann, 1994). The microvillous plasma membrane of the
Syncytiotrophoblast and basal plasma membrane are thought to be the most important membrane of the placental barrier as they represent the rate limiting step in the transport process. Among the substrates the fetus requires, amino acids seems to be the important determinant in fetal growth. There are several amino acid transports in the microvillous plasma membrane of the placenta, but the system A amino acid transporter, which transports non-essential neutral amino acids, has drawn the most attention. In 1988, Dicke and Henderson first described a defect of system A amino acid transport in FGR pregnancies (Dicke and Henderson, 1988). Later studies demonstrated that reduced activity or expression of system A amino acid transporter was associated with FGR (Cramer et al., 2002; Mahendran et al., 1993), and also related to the severity of FGR (Glazier et al., 1997). Consistent with an effect on placental transport, maternal GH treatment increases placental capacity for simple diffusion and stimulates fetal growth (Harding et al., 1997; Jenkinson et al., 1999), and increases fetal body weight and length in sheep FGR models after placental embolization (de Boo et al., 2008). Another animal study observed increased placental nutrient transporter expression after maternal GH intervention, not accompanied by alterations of the placental structure (Tung et al., 2012). These finding suggests that GH-V may influence placental nutrient transport, in addition to altering the blood flow and placental morphology.

**Gestational diabetes mellitus**

Insulin resistance is one of characteristic feature of GDM and GH-V may play a role in the development of insulin resistance during diabetic pregnancy. Animal studies have demonstrated that GH-V induces insulin resistance by increasing fasting and postprandial hyperinsulinemia (Barbour et al., 2002). In human studies, higher levels of circulating GH-V has also been observed in diabetic pregnancies (Fuglsang et al., 2003;
McIntyre et al., 2000). As both the GHR and insulin receptor activate PI3-K and MAPK signalling pathways, GH-V may interact with insulin receptor signalling and cause insulin resistance via specific signalling components (i.e. IRSs) (Barbour et al., 2004; Smith et al., 1997; Souza et al., 1994). However, their correlation is still unclear and needs further clarification.

**Pre-eclampsia**

As described in Section 1.4.2., impaired trophoblast invasion and placental angiogenesis are key pathogenic mechanisms of PE (Kaufmann et al., 2003; Redman and Sargent, 2005). As GH-V influences the process of placentation, PE is thought to be another disorder of pregnancy possibly associated with aberrant GH-V. However, the results of studies are conflicting. Papadopoulou et al. analysed samples in pairs of serum and amniotic fluid from 25 PE combined with FGR patients at 16-22 weeks of gestation (Papadopoulou et al., 2006). They found that GH-V concentrations in both serum and amniotic fluid were higher in pregnancies complicated by PE and FGR. A cross-sectional study observed that maternal circulating concentrations of GH-V at 20-42 weeks gestation in patients with PE was higher than normal pregnant women, and patients with PE and SGA had lower maternal serum concentrations of GH-V than preeclamptic patients without SGA (Mittal et al., 2007a). Contrary to their findings, Sifakis et al. observed that no differences of maternal serum GH-V concentration at first trimester in a case control study from 60 PE cases and 120 controls (Sifakis et al., 2011b). At the molecular level, Mannik et al. found that the placental expression profile of entire GH gene cluster in PE pregnancies showed a trend towards reduced expression compared to the control and the reduced GH-V expression was statistically significant (Mannik et al., 2012).
Other conditions

Additional studies have demonstrated that the maternal serum and amniotic concentrations of GH-V were increased in pregnancies affected by chromosomal anomalies, such as Down syndrome, compared with controls (Moghadam et al., 1998; Papadopoulou et al., 2008; Sifakis et al., 2009). GH-V may be an additional marker in Down syndrome screening, apart from the triple test (hormones choriongonadotropin, α-foetoprotein and oestrogen). Adding GH-V to the screening test increases the detection rate of aneuploidy from 65.6 to 71.9 % (Baviera et al., 2004). In pregnancies affected by Down syndrome, some defects in placentation, especially the formation of syncytiotrophoblast layer, have been revealed in vitro (Massin et al., 2001). As GH-V is secreted in the syncytiotrophoblast, the expression of GH-V is diminished (Frendo et al., 2000), lower serum concentration of GH-V should be observed in Down syndrome pregnancies. However, the results of current studies are inconsistent. Early studies found that the GH-V levels of trisomy 21 affected pregnancies on second trimester are higher than the normal ones. A hypothesis has been established by Frendo et al. as an explanation for these discrepancies. They suggested that the increased maternal placental levels may be a consequence of post-transcriptional switch from glycosylated GH-V to a hyper glycosylated from which has a longer half-life (Frendo et al., 2000). Conversely, Sifakis et al. observed serum GH-V during first trimester was significantly lower in trisomy 21 compared to euploid pregnancies (Sifakis et al., 2010b). The role of GH-V in chromosomal aberrations awaits further elucidation.

GH, especially GH-V, is closely associated with human reproduction and fetal growth. GH-V may play an important role in the pathology of several pregnancy complications, in respect to fetoplacental blood supply, placental nutrient transport and the process of
placentation. However, the exact physical and pathological effect of GH-V in pregnancy is still largely unknown and needs further investigation.

1.6. Thesis objectives

Prompted by the evidence described above suggesting a potential role of GH-V in pregnancies, the aim of this project was to determine the effect of GH-V on pregnancy outcomes, in term of fetal growth and maternal metabolism, in mouse models and the relationship between circulating GH-V and complicated human pregnancies in a cohort.

The overall aims of the study were to:


b. Examine the dose-response effect of pulsatile versus continuous administration of recombinant GH-V in a normal mouse model.

c. Determine GH-V concentrations in maternal serum samples in complicated human pregnancies.
Chapter 2: Methodology

2.1. Materials

2.1.1. Chemicals, reagents and antibodies

Table 2.1: List of chemicals, reagents and suppliers

<table>
<thead>
<tr>
<th>Material</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide/Bis solution (40%)</td>
<td>Bio-Rad laboratories, Inc., Hercules, CA, USA</td>
</tr>
<tr>
<td>Agarose (Ultra-pure)</td>
<td>Life Technologies, Carlsbad, CA, USA</td>
</tr>
<tr>
<td>Ammonium persulfate (APS)</td>
<td>Serva Electrophoresis, Heidelberg, Germany</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Sigma Chemical Company, St Louis, MO, USA</td>
</tr>
<tr>
<td>Bromophenol Blue</td>
<td>Sigma Chemical Company, St Louis, MO, USA</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>Immuno Chemical Products Ltd, Auckland, New Zealand</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Scharlau Chemie SA, Barcelona, Spain</td>
</tr>
<tr>
<td>cOmplete, Mini, EDTA-free protease inhibitor</td>
<td>Roche Diagnostics GmbH, Mannheim, Germany</td>
</tr>
<tr>
<td>Dimethyl-sulphoxide (DMSO)</td>
<td>Sigma Chemical Company, St Louis, MO, USA</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate</td>
<td>Sigma Chemical Company, St Louis, MO, USA</td>
</tr>
<tr>
<td>Dithiothreitol (DTT)</td>
<td>Sigma Chemical Company, St Louis, MO, USA</td>
</tr>
<tr>
<td>DC protein assay reagents</td>
<td>Bio-Rad laboratories, Inc., Hercules, CA, USA</td>
</tr>
<tr>
<td>DMEM (high glucose) media</td>
<td>Life Technologies, Carlsbad, CA, USA</td>
</tr>
<tr>
<td>DMEM (low glucose) media</td>
<td>Life Technologies, Carlsbad, CA, USA</td>
</tr>
<tr>
<td>EDTA</td>
<td>Sigma Chemical Company, St Louis, MO, USA</td>
</tr>
<tr>
<td>Ethanol (absolute, analytical grade)</td>
<td>Scientific Supplies Ltd, Auckland, New Zealand</td>
</tr>
<tr>
<td>Ethidium bromide</td>
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</tr>
<tr>
<td>Fetal bovine serum (FBS)</td>
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<td>Glycerol</td>
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<tr>
<td>Glycine</td>
<td>Applichem GmbH, Darmstadt, Germany</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>Scientific Supplies Ltd, Auckland, New Zealand</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Scientific Supplies Ltd, Auckland, New Zealand</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>Gibco New Zealand Ltd., Auckland, New Zealand</td>
</tr>
<tr>
<td>Magnesium Chloride</td>
<td>Scientific Supplies Ltd, Auckland, New Zealand</td>
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<tr>
<td>Methanol</td>
<td>Scientific Supplies Ltd, Auckland, New Zealand</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>Sigma Chemical Company, St Louis, MO, USA</td>
</tr>
<tr>
<td>Penicillin (1000 U/mL)</td>
<td>Gibco New Zealand Ltd., Auckland, New Zealand</td>
</tr>
<tr>
<td>Streptomycin (1000 µg/mL)</td>
<td>Gibco New Zealand Ltd., Auckland, New Zealand</td>
</tr>
<tr>
<td>PVDF membrane</td>
<td>Bio-Rad laboratories, Inc., Hercules, CA, USA</td>
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<tr>
<td>RPMI 1640 medium</td>
<td>Life Technologies, Carlsbad, CA, USA</td>
</tr>
<tr>
<td>SeeBlue plus2 protein marker</td>
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</tr>
<tr>
<td>Sodium dodecyl sulfate (SDS)</td>
<td>Life Technologies, Carlsbad, CA, USA</td>
</tr>
<tr>
<td>Sodium Dihydrogen Phosphate</td>
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<tr>
<td>Supersignal WestDura extended duration substrate</td>
<td>Pierce Biotechnology, Inc., Rockford, Illinois, USA</td>
</tr>
<tr>
<td>Tetramethylethylendiamine (TEMED)</td>
<td>Sigma Chemical Company, St Louis, MO, USA</td>
</tr>
<tr>
<td>Tris</td>
<td>Serva Electrophoresis, Heidelberg, Germany</td>
</tr>
<tr>
<td>Triton X100</td>
<td>Sigma Chemical Company, St Louis, MO, USA</td>
</tr>
<tr>
<td>Trizol</td>
<td>Life Technologies, Carlsbad, CA, USA</td>
</tr>
<tr>
<td>Antibody</td>
<td>Catalogue Number</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Anti-rabbit IgG, HRP-linked Antibody</td>
<td>7074</td>
</tr>
<tr>
<td>Anti-mouse IgG, HRP-linked Antibody</td>
<td>A4416</td>
</tr>
<tr>
<td>Anti-goat IgG, HRP-linked Antibody</td>
<td>A5420</td>
</tr>
<tr>
<td>Novex™ Phospho-STAT5 pTyr694 Antibody</td>
<td>44-390G</td>
</tr>
<tr>
<td>Monoclonal Anti-β-Actin antibody produced in mouse</td>
<td>A1978</td>
</tr>
<tr>
<td>Stat5 Antibody (C-17)</td>
<td>sc-835</td>
</tr>
<tr>
<td>Placental Growth Hormone Antibody 78.7C12</td>
<td>MCA5828G</td>
</tr>
<tr>
<td>Placental Growth Hormone Antibody 78.8E8</td>
<td>MCA5827G</td>
</tr>
</tbody>
</table>

Table 2.3: List of recombinant human hormones and suppliers

<table>
<thead>
<tr>
<th>Recombinant human hormones</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human placental growth hormone (22 kDa)</td>
<td>Protein Laboratories Rehovot, Rehovot, Israel</td>
</tr>
<tr>
<td>Human pituitary growth hormone (22 kDa)</td>
<td>Harbor-UCLA Medical Centre, Torrance CA, USA</td>
</tr>
<tr>
<td>Human placental lactogen antigen</td>
<td>Harbor-UCLA Medical Centre, Torrance CA, USA</td>
</tr>
</tbody>
</table>

### 2.1.2. Additional chemicals

The human GHR antagonist B2036 was provided in liquid form by Pfizer at 10.4 mg/ml (472.73 µM) and stored in aliquots at -20°C.

### 2.2. Animals

All protocols were approved by the Animal Ethics Committee of the University of Auckland. Male and female C57BL/6J (B6) mice (Jackson Laboratories) were housed under standard conditions and maintained at 22°C with a 12h light/dark cycle and with ad-libitum access to food and water. Animals were group housed whenever possible to minimise isolation stress.
2.2.1. Timed-mating

Timed matings were conducted at 6 to 8 weeks of age when females had reached full sexual maturity. 1 to 3 females were placed into a clean cage overnight with a selected male breeder. The presence of a vaginal plug denoted day 0.5 of gestation. Experiment time-points included day 12.5 (mid to late gestation, establishment of feto-maternal circulation), day 18.5 (late gestation, one day prior to normal term delivery).

2.2.2. Osmotic pump

Osmotic pump preparation

Alzet osmotic pumps were purchased from Durect Corporation (Cupertino, CA, USA). Pump selection was based on the size, duration and flow rate. Model 1007D (100 µl) was designed to release its contents at a rate of 0.5 µl/h over one week duration. The concentration of the drug solution was determined by the mass delivery rate and the pumping rate of the pump. Recombinant human GH-V was reconstituted in 0.4% NaHCO₃ adjusted to pH 9. After filling, the pump was placed in sterile 0.9% saline at 37°C overnight for priming. To ensure that the recombinant proteins would remained active over the experiment, activity was tested at 37°C using an AlphaScreen assay (details in Chapter 3).

Osmotic pump implantation

At day 12.5, pregnant mice were anesthetized by isoflurane inhalation. Hair on the back was removed by shaving and the skin was disinfected with a 70% methanol isopropyl alcohol swab. A mid-scapular incision was made and a pocket for the pump created by inserting a hemostat into the incision. The filled pump was inserted into the pocket, and the wound was closed with sutures. Delivery was verified by measurement of the residual volume in the pump reservoir after explanation.
2.2.3. Evaluation of maternal and fetal growth

Maternal body weight and food intake

Animal body weight was measured every three days before mating. Once a vaginal plug presented, measurements were taken every two days from gestational day 0.5 to day 10.5 and then every day from day 10.5 to day 18.5. On day 10.5, pregnant mice were rehoused as in pairs where possible, or as singletons. A fixed amount of food (100g) was given on day 10.5 and maternal food intake per day was calculated by measuring the weight remaining from food pre-weighed the previous day. Animals were maintained on a standard commercially sourced chow diet (3.1kcal/g).

Table 2.4: Composition of the standard chow diet (Harlan Teklad Global Diet 2018)

<table>
<thead>
<tr>
<th></th>
<th>Harlan Teklad Global Diet 2018</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories from protein</td>
<td>24%</td>
</tr>
<tr>
<td>Calories from fat</td>
<td>18%</td>
</tr>
<tr>
<td>Calories from carbohydrate</td>
<td>58%</td>
</tr>
<tr>
<td>Energy Density (kcal/g)</td>
<td>3.1</td>
</tr>
</tbody>
</table>

**Macronutrients:**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>18.6%</td>
</tr>
<tr>
<td>Fat</td>
<td>6.2%</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>44.2%</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>3.5%</td>
</tr>
<tr>
<td>Neutral detergent fibre</td>
<td>14.7%</td>
</tr>
<tr>
<td>Ash</td>
<td>5.3%</td>
</tr>
</tbody>
</table>

Fetal growth

On day 18.5, fetal wet weights were measured after blotting following laparotomy. Fetal anthropometric measurements, including crown-rump length (from top of head to start of the tail following the curve of the spine), and abdominal circumference (taken from where the umbilical cord inserts), were performed by a single observer using cotton thread.
2.2.4. **Blood and tissue collection**

On day 12.5 and day 15.5, maternal blood sample was taken from tail tip using heparin coated capillary tubes (Microvette CB 300, Sarstedt) and immediately stored on ice until later processing. On day 18.5, pregnant mice were fasted for 6h (8am-2pm), and killed by cervical dislocation. A blood sample was collected by cardiac puncture.

Blood samples were centrifuged at 2500rpm for 10 min at 4°C. Plasma supernatants were aliquoted and stored at -80°C until subsequent analysis – multiple aliquots were taken to avoid potential effects of freeze-thaw cycles and stored at -80°C.

Following laparotomy, maternal liver, kidneys, spleen, pancreas, perirenal fat pads, retroperitoneal fat pads, gonadal fat pads, as well as placentas were dissected and weighted. Samples were either snap frozen in liquid nitrogen and stored at -80°C, and or fixed in 10% neutral buffered formalin.

2.3. **Myography**

Following euthanasia, the uterus was removed and placed in ice-cold physiological saline solution (PSS; mmol/L; 119 NaCl, 25 NaHCO₃, 4.69 KCl, 2.4 MgSO₄, 1.6 CaCl₂, 1.18 KH₂PO₄, 5 glucose, 0.034 EDTA; pH 7.4). The main uterine artery was carefully dissected. Arterial segments of approximately 2 mm in length were then cut and mounted in an isometric wire myograph system (610M wire myography; Danish Myotechniques, Aarhus, Denmark). The vessels were bathed in 5 mL PSS, gassed with 5% CO₂/95% air, maintained at a temperature of 37°C, and allowed to equilibrate for 30 min before normalization to an internal diameter of 0.9 of L13.3kPa using normalization software (Myodata, Danish Myotechnologies, Aarhus, Denmark). This corresponds to a transmural pressure of ~ 90 mmHg. Following normalization, vessels were constricted with phenylephrine (PHE; 10⁻⁵ mol/L) twice, with a 15 min wash
between activation periods. The constriction was allowed to plateau, then vessels were relaxed with the endothelial dependent vasodilator acetylcholine (ACh; 10^{-5} \text{ mol/L}) at the end of the second PHE-induced contraction in order to confirm the viability and endothelial integrity. Vessels that failed to respond to either PHE or ACh were rejected. After washing with PSS repeatedly and resting for 30 min, dose–response curves to PHE (10^{-10}-10^{-5} \text{ mol/L}) were then constructed. Each dose was added at 2 min intervals or after the constriction reached a plateau. Vessels were washed with PSS twice and allowed to rest for 30min. Following incubation with GH-V (500 ng/ml) for 30min, a second dose–response curve to PHE (10^{-10}-10^{-5} \text{ mol/L}) was performed. The effective concentration 80 (EC80) was calculated for individual vessel segments before and after GH-V treatment. At the end of the experiment, the vessels were washed twice with PSS. PSS was then replaced with 5ml high-potassium PSS (KPSS, mmol/L; 12.45 NaCl, 25 NaHCO₃, 120 KCl, 2.4 MgSO₄, 1.6 CaCl₂, 1.18 KH₂PO₄, 5 glucose, 0.034 EDTA; pH 7.4), and the constriction allowed to plateau.

Data were continuously collected and the vessel active wall tension (ΔT in mN/mm) was transformed to active effective pressure [ΔT/(diameter/2000)] denoted by kPa.

### 2.4. Biochemical and molecular analysis

#### 2.4.1. AlphaScreen assay

AlphaScreen is a bead-based chemistry used to study biomolecular interactions in a microplate format. AlphaScreen SureFire p-STAT5 (PerkinElmer, US) was used to measure the phosphorylation of endogenous STAT5A and STAT5B induced by GH-N and GH-V in cellular lysates from BA/F3-GHR cells which provide a useful model to test GH activity. Lysates were normalised using a GAPDH AlphaScreen assay. Cells were plated in a 96 well culture plate and incubated in serum-containing media overnight. Following serum starvation for 24 h, cells were treated with 500 ng/ml GH-
V for 10 min (detailed in Chapter 4). Cells were lysed using 50ul 1 X lysis buffer at room temperature with shaking (~350 rpm) for 10 min. Lysates were frozen and stored at -80°C for later analysis. 4 ul lysate was transferred to a 384 well proxiplate plate (neat for p-STAT5; 1/10 dilution for GAPDH). 5 ul acceptor bead mix (1 ul Activation buffer + 4 ul Reaction buffer) was added to each well, followed by 2 h incubation. 2 μL of donor mix was added under subdued light and then incubated for an additional 2 h at room temperature. AlphaScreen signal (counts) were read on an EnVision Multilabel plate reader (PerkinElmer). The averaged counts for untreated and treated cells were calculated and p-STAT5 readings were normalized to GAPDH. p-STAT5 dose response curves were analysed using 4 parameter non-linear regression to assess EC50, Min and Max signals, and Hillslope factors.

2.4.2. Blood analysis

Commercially sourced enzyme-linked immunosorbent assay (ELISA) kits were used to measure the concentration of insulin and IGF-1 in mouse plasma and IGF-1, IGF-2, IGFBP-1 and IGFBP-3 in human serum. Species specific kits were utilised where available. Each kit was performed as per the manufacturer’s provided protocol. In general, blood samples were thawed on ice and centrifuged for 3 min at 2500rpm at 4 °C prior to performing analysis to remove fibrous clots. When multiple plates were needed for one marker, control samples were included on each plate to control for time-of-day and inter-assay effects. All intra- and inter-assay coefficients of variation were < 5%.

The source of ELISA kit each analyte measured is summarised in the following table. (Table 2.5)
Table 2.5: Biochemical assay information

<table>
<thead>
<tr>
<th>Name</th>
<th>Assay type</th>
<th>Manufacturer</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Insulin</td>
<td>ELISA</td>
<td>CrystalChem, USA</td>
<td>90080</td>
</tr>
<tr>
<td>Mouse IGF-1</td>
<td>ELISA</td>
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<td>E25</td>
</tr>
<tr>
<td>Human IGF-1</td>
<td>ELISA</td>
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<td>Human IGFBP-3</td>
<td>ELISA</td>
<td>Mediagnost, Germany</td>
<td>E03A</td>
</tr>
</tbody>
</table>

2.4.3. RNA extraction

Liver samples from female mice were collected from the left lobe of the liver and crushed into small pieces in liquid nitrogen using a mortar and pestle. Approximately 20 mg liver tissue was added to 1 ml Trizol in 2 ml Eppendorf Safe-Lock Tubes and homogenized using a tissue lyser (TissueLyser II, Qiagen, US). All equipment that physically contacted tissue was cleaned and freshly autoclaved before use, and was wiped several times with isopropyl alcohol between samples to avoid any cross-contamination. Tissue lysates in Trizol were incubated at room temperature (15-30°C) for 5 min to permit the complete dissociation of nucleoprotein complexes. 0.2 ml chloroform was added per 1 ml Trizol. Tubes were shaken vigorously by hand for 15 sec, incubated for 3 min at room temperature and centrifuged at 14000 g for 15 min at 4 °C. The aqueous phase (top layer) was transferred to a fresh 1.5m eppendorf tube. Taking care not to disturb the phenol layer, the aqueous phase was approximately 60% of the original Trizol volume used. A second extraction step with chloroform was performed to remove any traces of phenol, and the supernatant was transferred to a fresh tube. RNA was precipitated by adding 0.5 ml isopropanol per ml Trizol. Incubating at 4°C for 20 min and centrifuging at 14000g for 15 min at 4°C, pellets were
washed twice with 75% ethanol and air dried for 15 min. Pellets were dissolved in DEPC H₂O.

2.4.4. RNA quantity and quality evaluation

Nanodrop
The concentration of all RNA samples was obtained by Nanodrop ND-1000 by measuring the spectrophotometric absorbance at 260 nm (A₂₆₀). The ratio of absorbance at 260 nm and 280 nm (A₂₈₀) was used as the primary criteria to assess the purity of RNA. A ratio of ~2.0 is generally accepted as “pure”. The ratio of absorbance at 260 nm and 230 nm (A₂₃₀) was used as a secondary measure of nucleic acid purity. The A₂₆₀/A₂₃₀ values for “pure” nucleic acid are often higher than the A₂₆₀/A₂₈₀. In this study, an A₂₆₀/A₂₈₀ ratio between 1.9-2.1 and A₂₆₀/A₂₃₀ 1.9-2.2 was accepted as appropriate for further experimental analysis.

Agilent 2100 Bioanalyzer
RNA quality control in selected samples was also carried out by using an Agilent Bioanalyzer 2100 according to the manufacturer’s instruction. The Bioanalyzer uses a chip to perform capillary electrophoresis and uses a fluorescent dye that binds to RNA to determine RNA concentration and integrity. A RNA Integrity Number (RIN) ranging from 1 to 10 was calculated by the software to indicate the integrity of RNA. A score of 1 means the sample has completely degraded whereas 10 indicates the sample is intact. All samples tested with the Bioanalyzer had a RIN ≥ 8 which is indicative of high quality RNA.

2.4.5. cDNA synthesis
Isolated RNA was treated with Deoxyribonuclease I (DNase I) (Life Technologies, Carlsband, USA) to eliminate DNA before transcription to cDNA. 1 µg RNA sample was
mixed with DEPC water, 1 µl 10X DNase I Reaction Buffer and 1 µl DNase I (supplied in kit) to make up 10 µl reaction volume in an RNase-free, 0.5 ml microcentrifuge tube on ice. Samples were incubated at room temperature for 15 min. The DNase I was inactivated by the addition of 1 µl of 25 mM EDTA solution to the reaction mixture and heated for 10 min at 65°C. The RNA sample was then ready to use in reverse transcription reactions.

Total RNA was reverse transcribed into cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). 1 µg RNA sample was mixed with water and 2 µl Random Hexamer Primer (supplied in the kit) to make a total of 13 µl Template-Primer Mix. RNA samples were denatured at 65°C for 10 min. 4 µl Transcriptor Reverse Transcriptase Reaction Buffer, 0.5 µl Protector RNase Inhibitor, 2 µl Deoxynucleotide Mix and 0.5 µl Transcriptor Reverse Transcriptase were added per tube. The reaction was incubated as follows: 1st step: 50°C for 60 min, 2nd step: 85°C for 5 min. Reverse transcribed cDNA was stored at -20°C for quantitative polymerase chain reaction (PCR).

2.4.6. Real time PCR
Real time PCR was carried out using predesigned probe-based PrimeTime qPCR assays (Integrated DNA Technologies, USA) on a Lightcycler 480 (Roche, Mannheim, Germany). Amplified fragments were checked for correct size by gel electrophoresis on a 3% agarose gel (see Section 2.4.8). mRNA levels were normalized to 3 housekeeping genes: Gapdh, β-Actin and Cox4i1 by subtracting the geometric mean Ct of housekeeping genes from the Ct for the gene of interest to produce a ΔCt value. The ΔCt for each treatment sample was compared with the mean ΔCt for vehicle-treated samples using the relative quantification 2-ΔΔCt method to determine fold-change.
All assays were validated for amplification efficiencies before experiments were carried out. This was achieved by performing a series dilution standard curve for each candidate gene. Standard curves points were repeated in triplicate to improve statistical significance and reliability. The CT value of each dilution point (Y) was plotted against log of the dilution factor (X) and a linear standard curve was generated. The slope of the standard curve was used to calculate the amplification efficiency (E) using the following formula:

$$E = 10^{-1/\text{slope}}$$

### 2.4.7. Semi-quantitative reverse transcription PCR

Semi-quantitative reverse transcription PCR (RT-PCR) was performed using KAPA SYBR FAST Universal One-Step qRT-PCR Kit (Kapa Biosystems, MA, USA). RT-PCR reactions were carried out on the Bio-Rad Thermal Cycler (Bio-Rad Laboratories, New Zealand). A no template control was included in each experiment. Each reaction started with 5 min reverse transcription at 42°C followed by enzyme inactivation for 3 min at 95°C. The PCR cycling consisted of 3 steps including denaturation at 95°C for 3 sec, annealing at specified temperature for 30 sec, and for the indicated number of cycles. \textit{GAPDH}, \textit{\( \beta \)-ACTIN} or \textit{18S r} served as a loading control.

Amplified reactions were separated on an agarose gel prepared in Tris-Acetate-EDTA buffer (TAE) containing 1% ethidium bromide. The RT-PCR product was mixed with KAPA DNA loading dye (6X) (Kapa Biosystems, MA, USA) and loaded onto the gel. A 1 kb plus DNA ladder (Life Technologies, Carlsband, CA, USA) served as a molecular weight marker. Gel electrophoresis was carried out in 1X TAE buffer at 80-100 V for 20-40 min. Bands were visualized under UV light, photographed and analysed.
Table 2.6: Reaction components for one-step RT-PCR

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR-grade water</td>
<td>Up to 20</td>
</tr>
<tr>
<td>2X KAPA SYBER FAST Master Mix</td>
<td>10</td>
</tr>
<tr>
<td>10µM Forward Primer</td>
<td>0.4</td>
</tr>
<tr>
<td>10µM Reverse Primer</td>
<td>0.4</td>
</tr>
<tr>
<td>50X KAPA RT Mix</td>
<td>0.4</td>
</tr>
<tr>
<td>Template RNA</td>
<td>As required (100 ng)</td>
</tr>
</tbody>
</table>

Table 2.7: Primer information (Self-designed)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mGhr</td>
<td>NM_010284</td>
<td>5’-GCTACTCTTTGGCAAAGCTTC 5’-CGTTGGCTTTCCCTTTTAGC</td>
</tr>
<tr>
<td>hPRLR</td>
<td>NM_000949</td>
<td>5’-TTTGCCCTCCAGCAGGGAACA 5’-CGCGAACGGTCGGTAAAATC</td>
</tr>
<tr>
<td>mPrlr</td>
<td>NM_011169</td>
<td>5’-TTTTGCACATGAACCCTGAA 5’-ACCAGCAGGTGAATGTTTCC</td>
</tr>
<tr>
<td>hGAPDH</td>
<td>NM_002046</td>
<td>5’-TGCACCACCAACTGCTTAGC 5’-CCCATGGACTGTGGTCATGAC</td>
</tr>
<tr>
<td>mGapdh</td>
<td>NM_001289726</td>
<td>5’-CTTTGGCATTGTGGAAGGCC 5’-CAGGGATGATGTTCTGGGCA</td>
</tr>
</tbody>
</table>
Table 2.8: Primer information (IDT PrimeTime qPCR Primer Assays)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Catalogue no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insr</td>
<td>Mm.PT.58.9275253</td>
</tr>
<tr>
<td>Pikca</td>
<td>Mm.PT.56a.11667792</td>
</tr>
<tr>
<td>Igf1</td>
<td>Mm.PT.58.32726889</td>
</tr>
<tr>
<td>IGF1</td>
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</tr>
<tr>
<td>Igfbp3</td>
<td>Mm.PT.58.6744601</td>
</tr>
<tr>
<td>Als</td>
<td>Mm.PT.58.6448982</td>
</tr>
<tr>
<td>Akt1</td>
<td>Mm.PT.58.8333433</td>
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<tr>
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</tr>
<tr>
<td>Akt3</td>
<td>Mm.PT.58.13175174</td>
</tr>
<tr>
<td>Cox4i1</td>
<td>Mm.PT.58.11169461</td>
</tr>
<tr>
<td>COX4I1</td>
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</tr>
<tr>
<td>Pik3r1</td>
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</tr>
<tr>
<td>Ghr</td>
<td>Mm.PT.58.29016393</td>
</tr>
<tr>
<td>Irs1</td>
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</tr>
<tr>
<td>Slc2a1</td>
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</tr>
<tr>
<td>Slc2a4</td>
<td>Mm.PT.58.32667331</td>
</tr>
<tr>
<td>Actb</td>
<td>Mm.PT.58.33257376.gs</td>
</tr>
<tr>
<td>Gapdh</td>
<td>Mm.PT.39a.1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Hs.PT.39a.22214836</td>
</tr>
</tbody>
</table>

2.5. Cell culture

2.5.1. General cell culture methods

All tissue culture work was undertaken in a laminar flow hood under sterile conditions.

2.5.2. Cell lines

The human prostate carcinoma cell line, LNCaP, hepatocellular carcinoma cell line, HepG2, and mouse myoblast cell line, C2C12, were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA).

2.5.3. Passaging and harvesting of cell lines

LNCaP cells were maintained in RPMI media (Gibco). C2C12 and HepG2 cells were maintained in DMEM media (Gibco). All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin.
and 2 mM L-glutamine. Cell lines were grown in 75 cm² tissue culture treated flasks (Greiner Bio-One, Frickenhausen, Germany) containing approximately 20 mL of growth media at 37°C in a humidified 5% CO2 incubator.

A continual stock of cell lines was maintained by splitting a portion of cells into a new flask once cells reached 70-80% confluence. To passage cells, the media was removed and cells were washed with PBS followed by addition of 1 mL of Trypsin/EDTA. Cells were incubated in the incubator for 3-5 min until all cells detached from the flask, which was verified under the microscope. Following trypsinisation, 15 mL of serum-supplemented media was added to each flask to neutralize the trypsin. The cell suspensions were transferred to 50 ml Falcon tubes and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and cells were resuspended in fresh media. For further culturing of stock cultures, an appropriate proportion of this media was transferred into a fresh tissue culture flask and approximately 20 ml of fresh media added, after which the cells were maintained at 37°C in a humidified 5% CO2 incubator.

2.5.4. Cell counting

For setting up experiments and measuring experimental end points, cell concentrations were measured using haemocytometer. 20 µl of the cell suspension was transferred to an Eppendorf tube and mixed with 180 µl of 0.4% Trypan blue dye. 10 µl of this dilution was transferred onto a haemocytometer and counted. The number of cells in 4 corner quadrants was counted and the number of cells/ml calculated using the formula below. This was repeated 3 times and value averaged.

\[
\text{Cells/ml} = \left( \frac{\text{Number of cells in 4 quadrants}}{4} \right) \times \text{dilution factor} \times 10000
\]
2.5.5. **Storage of cell lines**
Cells were trypsinised and resuspended in 10 ml of fresh media, counted and then centrifuged (1000 rpm, 5 min). Following removal of the supernatant, cells were resuspended in freezing media (40% FBS, 10% DMSO) at $5 \times 10^6$ – $1 \times 10^7$ cells/ml and 1 ml aliquots of the cell suspension were placed into cryogenic vials (Nalgene, Rochester, NY, USA). The vials were placed into a freezing chamber containing isopropanol (Nalgene, Rochester, NY, USA) and placed into a -80°C freezer for 24 h to allow gradual cooling and freezing for cell preservation. After 24 h frozen cells were stored in the vapour phase of liquid nitrogen for long-term storage.

2.5.6. **Revival of cell lines from liquid nitrogen storage**
Cell aliquots from each cryogenic vial were thawed immediately in 10 ml of 37°C serum-supplemented culture media, transferred into a 25 cm$^2$ tissue culture flask (Greiner Bio-One, Frickenhausen, Germany) and cultured at 37°C in a humidified 5% CO$_2$ incubator. The media was changed the next day (to remove DMSO and dead cells) and then every two days to allow growth of revived cells.

2.5.7. **Cell culture procedures for RNA and protein extraction**
An appropriate number of cells was plated in a 10 cm or 6-well tissue culture treated plate containing serum-replete media (10% FBS) and incubated for 24 h to allow attachment of cells to the culture plate. After incubation, the media was removed and cells were washed twice with PBS to wash out residual serum-supplemented media. Serum-free media was added to cells and cells were incubated overnight before GH treatment.

**RNA extraction**
Total RNA was extracted using Trizol as described above.
Protein extraction

Following relevant treatment, media was removed and cells were washed twice with ice-cold PBS. Protease inhibitor solution, was prepared by dissolving one Complete Mini protease inhibitor cocktail tablet (Roche Applied Science, Penzberg, Germany) into 1.5 ml of MilliQ water, and was mixed with 8.5 ml of cell lysis buffer. 100 μl or 500 μl of this buffer was added to each well of the 6-well culture plate or 10 cm tissue culture plate, respectively, and plates were kept on ice for 30 min. Cells were then scraped off the plates using disposable plastic pipette tips and cell lysates were transferred into pre-chilled Eppendorf tubes. Cell lysates were homogenised by pipetting and then centrifuged for 10 min at 4°C using 5417R Eppendorf Centrifuge (Eppendorf AG, Hamburg, Germany). Supernatants were transferred into new tubes for quantification.

Table 2.9: Lysis buffer recipe

<table>
<thead>
<tr>
<th>Lysis buffer</th>
<th>7.5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% Triton X-100</td>
<td>1 ml</td>
</tr>
<tr>
<td>150 mM NaCl</td>
<td>15 μl</td>
</tr>
<tr>
<td>10% glycerol</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>50 mM Tris-HCL</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>2 mM EDTA</td>
<td>40 μl</td>
</tr>
<tr>
<td>100 mM NaF,</td>
<td>1 ml</td>
</tr>
<tr>
<td>1 mM sodium orthovanadate,</td>
<td>50 μl</td>
</tr>
<tr>
<td>H2O</td>
<td>3.395 ml</td>
</tr>
</tbody>
</table>

Protein quantification

Protein concentrations were measured using DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). 1 mg/mL BSA was diluted with lysis buffer to generate a series of standards containing from 0 to 5 mg/mL protein. Standards and unknown samples (5 μl) were transferred into a 96-well microtiter plate. Reagent A (25 μl) was added into each well, followed by 200 μl of reagent B. The microtiter plate was
incubated for 30 min with gentle agitation at room temperature in the dark. Absorbances were read at 750 nm using Synergy2 Multi-mode Microplate reader and Gen5 data analysis software (BioTek). Based on the absorbances of the standards, a standard curve was plotted and the concentration of unknown proteins was calculated. Following this the samples were stored at -80°C.

2.5.8. Western blot procedure

Protein separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)
SDS-PAGE gels (4% stacking gel and 7.5 separating gel) were prepared. Samples were mixed with 6X SDS sample buffer (5% mercaptoethanol) and heated at 70°C for 10 min to denature and reduce the proteins. 20-30 µl samples were loaded on the gel and the gel was run for approximately 60 min at 100 V (const.) until the dye front had reached the bottom of the glass plate.

Protein transfer
Following electrophoresis, a Polyvinylidene Fluoride (PVDF) membrane was soaked in methanol for 30 sec, washed with water and equilibrated in transfer buffer (14.4 g Glycine; 3 g Tris; 200 ml Methanol; add H2O to 1 L) for 15 min with light agitation. The transfer apparatus was assembled in the following order: black side of the gel holder cassette; pre-wetted fibre pad; pre-wetted filter paper; gel; membrane; pre-wetted filter paper; pre-wetted fibre pad; red side of the gel holder cassette. Transfer was carried out for 90 min at 100 V (const.) and 400 mA. After the transfer, the PVDF membrane was removed from the transfer apparatus and incubated in blocking buffer (5% no fat milk powder/PBS-T (0.1% Tween-20)) for 1 h at room temperature on a rocker with agitation.
Antibody incubation and detection of proteins

After blocking, the membrane was incubated overnight at 4°C in primary antibody diluted in blocking buffer according to the manufacturer’s datasheet. Afterwards, the membrane was washed 3 times for 10 min in wash buffer (PBS-0.1% Tween-20) and incubated in corresponding secondary Horseradish Peroxidase (HRP)-conjugated antibody diluted in blocking buffer for 1-2 h at room temperature. Then the membrane was washed 3 times with wash buffer. To detect the protein of interest the membrane was incubated in SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology Inc, Rockford, Illinois, USA), according to the manufacturer’s instructions. The membrane was drained of excess developing solution, wrapped in plastic wrap and exposed to X-ray film or analysed on a BioRad ChemiDoc MP System.

2.6. Statistical analysis

All statistical analyses in this thesis were conducted using SigmaPlot 12.0 (Systat software, CA, USA) or IBM SPSS Statistics 21 (Watson Analytics, NY, USA). Appropriate analysis methods were chosen based on the research questions, design of the experiment, nature of the variables and distribution of the data sets.

The graphical presentations were generated using GraphPad Prism 6 (GraphPad Software, CA, USA). All data are expressed as means ± SEM. Data were analysed using an unpaired two-tailed t-test, regression analysis or analysis of variance (ANOVA). * corresponds to p values less than 0.05, ** corresponds to p values less than 0.01.

Some experiments in this thesis are of a balanced 2 x 2 factorial designed, therefore data were analysis by two-way ANOVA if they meet the assumptions re parametric testing. Data that failed to meet the criteria required for parametric analysis were transformed to achieve normal distribution and equal variance. Where appropriate,
post-hoc analysis were performed (Tukey's procedure) to determine which groups were significantly different from each other.

Categorical and numerical variables were compared using chi-square or Fisher’s exact test. Pearson’s coefficient was used to determine correlations between variables, presented as r values. Linear regression analysis was used to explore the relationships between two variables. Forward stepwise regression models were adopted to determine independent variables predicting changes in dependant values.
Chapter 3: The effect of placental growth hormone in mice pregnancies

3.1. Preface

The following chapter contains data from a published article “The Placental Variant of Human Growth Hormone Reduces Maternal Insulin Sensitivity in a Dose-Dependent Manner in C57BL/6J Mice” (Appendix I). A version of this chapter was published in Endocrinology in 2016. Endocrinology.

In this chapter, a study was conducted to determine the dose-response relationship for human GH-V treatment in a mouse model of normal pregnancy.

3.2. Introduction

The GH and IGF-1 axis is a major regulator of mammalian growth. As discussed in Chapter 1, Two GH genes encode two 22 kDa GH proteins: pituitary GH (GH-N; GH1) and GH-V variant (GH-V; GH2). The protein sequences of GH-N and GH-V are highly conserved, differing by 13 out of 191 amino acids which are scattered throughout the protein (Alsat et al., 1998) but they have distinct expression profiles. GH-N is secreted in a pulsatile fashion from the pituitary, and is also expressed at extra-pituitary sites, while GH-V is secreted from the placenta in a nonpulsatile manner. The continuous secretion of GH-V into the maternal compartment is thought to contribute to maternal metabolic alterations during pregnancy (Eriksson et al., 1989). Both proteins bind the GHR with similar affinity and share similar physiological somatotrophic, lactogenic and lipolytic properties (Alsat et al., 1997; Verhaeghe, 2008). However, compared with
GH-N, GH-V binds the prolactin (PRL) receptor poorly and its lactogenic affects are greatly reduced (Igout et al., 1995; MacLeod et al., 1991b).

During pregnancy, concentrations of GH-N in the maternal circulation decline, whilst GH-V expression increases from week five, gradually replacing GH-N completely at approximately 20 weeks (Eriksson et al., 1989). The increase in maternal circulating GH-V is positively associated with fetal growth and circulating IGF-1 concentrations during pregnancy (Chellakooty et al., 2004; Koutsaki et al., 2011; McIntyre et al., 2000; Mirlesse et al., 1993). A growth-promoting effect for GH-V has been demonstrated *in vivo* in non-pregnant hypophysectomized rats treated with GH-V and transgenic mice (Barbour et al., 2002; Caufriez et al., 1990a; MacLeod et al., 1991b; Selden et al., 1988a). Moreover, GH-V has pro-angiogenic properties (Struman et al., 1999) and stimulates trophoblast invasion *in vitro* and may therefore play a role in the process of placentation (Corbacho et al., 2002; Lacroix et al., 2005).

One of the characteristic features of the maternal adaptation to pregnancy is insulin resistance with resultant hyperinsulinemia (Catalano et al., 1991). This environment ensures adequate nutrient supply to the fetus. However, increased insulin resistance can lead to gestational diabetes. Placental hormones, and to a lesser extent increased fat deposition during pregnancy, may contribute to insulin resistance during pregnancy (Catalano, 2010; Ryan and Enns, 1988). Consistent with this, higher concentrations of circulating GH-V have been observed in pregnancies complicated by diabetes (Fuglsang et al., 2003; McIntyre et al., 2000). Furthermore, GH-V has been demonstrated to induce severe insulin resistance and alter body composition in non-pregnant transgenic mice that overexpress GH-V (Barbour et al., 2002).
Despite a proposed role for GH-V during pregnancy, the effects of GH-V administration on metabolic parameters and outcomes related to maternal and fetal growth are poorly understood. In the present study, the activity of GH-V in human and mouse cell lines was investigated, and the dose-response relationship for recombinant GH-V administration examined in a mouse model of normal pregnancy.

3.3. Hypothesis and aims

The hypothesis is that exogenous GH-V administration impacts fetal growth and maternal metabolic outcomes in mice.

The aims of this study are therefore:

a. To confirm activity of recombinant GH-V in cell lines.

b. To determine the effect of GH-V treatment on fetal growth;

c. To determine the effect of GH-V treatment on maternal metabolic outcomes, including maternal growth, body composition, and insulin sensitivity;

d. To elucidate the underlying mechanisms.

3.4. The effect of human GH-V on mice

3.4.1. Activation of the mouse GHR by GH-V

As mice don't express placental growth hormone during pregnancy, experiments were conducted to confirm the activity of the human GH-V against the human and mouse GHR. The activation of STAT5 signal transduction was determined in human and mouse cell lines by Western blotting. Other studies have also shown that GH-V can activate the mouse GHR, but it was important to confirm this as the recombinant human GH-V used (from PLR Ltd) hadn’t been published before.
Both GH-N and GH-V stimulated STAT5 phosphorylation in the human prostate cancer cell line, LNCaP (Figure 3.1). GH can activate both the GH and PRL receptors. To determine whether GH-V activation of STAT5 occurred through binding to the GHR, PRL receptor expression was investigated. LNCaP cells have previously been demonstrated to only express very low levels of PRL receptor mRNA (22). I was unable to detect PRL receptor expression in LNCaP cells by semi-quantitative RT-PCR (Figure 3.2). Furthermore, induction of STAT5 phosphorylation by GH-N and GH-V was abrogated by the specific GHR antagonist, B2036, thus confirming that phosphorylation of STAT5 occurred through activation of the GHR (Figure 3.1). B2036 is the protein component of the pegylated peptide inhibitor, Pegvisomant (Pfizer). It is specific to the GHR and has no effect on the PRL receptor (van der Lely and Kopchick, 2006).

![Figure 3.1: Activation of GHR by GH-V in LNCaP cell line. Serum-starved LNCaP was treated with 500 nM B2036 for 30 min, followed by 500 ng/ml GH-N or GH-V for 10 min. Immunoblotting of cell lysates for phosphorylated STAT5 (pSTAT5) and total STAT5 was performed.](image-url)
Figure 3.2: PRL receptor expression in human cell lines. Expression of the human PRL receptor (hPRLR) by semi-quantitative RT-PCR in the prostate cancer cell line, LNCaP, and the breast cancer cell line T47D. GAPDH was used as a loading control.

Activation of the mouse GHR by recombinant human GH-V was confirmed in the mouse myoblast cell line, C2C12. Ghr and Prl receptor expression was detectable in C2C12 cells by semi-quantitative RT-PCR (Figure 3.3). Treatment with either GH-V or GH-N stimulated STAT5 phosphorylation in C2C12 cells (Figure 3.4 and 3.5). B2036 treatment reduced STAT5 activation by either GH-V or GH-N, thus confirming GH-V and GH-N both activated the mouse GHR (Figure 3.3). However, B2036 did not completely abrogate STAT5 phosphorylation. Residual STAT5 activity in this cell line may be due to activation of the mouse PRL receptor by GH-V and GH-N, as both have been previously demonstrated to activate the rodent PRL receptor (Igout et al., 1995; MacLeod et al., 1991b). Failure to completely abrogate activation may also be due to reduced affinity of B2036 for the mouse GHR.
Figure 3.3: *Ghr* and *Prl* receptor expression in mice. Expression of the *mouse Ghr* (*mGhr*) and the *mouse Prl receptor* (*mPrlr*) in mouse C2C12 cells, compared with RNA isolated from C57BL/6J (B6) liver (non-pregnant female) and full-term placenta. *Gapdh* was used as a loading control.

Figure 3.4: Activation of GHR by GH-V in C2C12 cell line. Serum-starved C2C12 was treated with 500 nM B2036 for 30 min, followed by 500 ng/ml GH-N or GH-V for 10 min. Immunoblotting of cell lysates for phosphorylated STAT5 (pSTAT5) and total STAT5 was performed.
Figure 3.5: Activation of GHR by GH-V in a dose-dependent manner. Serum-starved C2C12 cells were treated with the indicated concentrations of GH-V and GH-N for 10 min. Immunoblotting of cell lysates for pSTAT5 and total STAT5 was performed. β-ACTIN was assayed as a loading control.

3.4.2. The effect of GH-V treatment on the tone of uterine arteries in mice

An animal study was conducted to determine whether there was a direct effect of GH-V on vessel tone using wire myography. Eight wild type female mice were mated and culled at gestational day 18.5. The uterine arteries were dissected and 4 segments were mounted on the myograph. Wire myography was performed as described in Chapter 2. Constriction was measured using phenylephrine (PHE; $10^{-10}$ to $10^{-5}$M). After performing the first dose-response curve to PHE, vessels were incubated with GH-V (500 ng/ml) for 30 min, and then the second dose-response curve to PHE was performed in order to test the effect of GH-V on vessel tone in response to vasoactive agents. Representative raw data are demonstrated in Figure 3.6. The effective concentration 80 (EC80) was calculated for individual vessel segments before and after GH-V treatment.
Figure 3.6: Representative wire myography tracing of mice (pregnant) uterine artery PHE dose-respond curves pre and post GH-V incubation. Mice uterine arteries were exposed to incremental doses of PHE (10^{-10} – 10^{-5}M) as indicated by the staircase annotation. Arteries were washed to baseline tension and incubated with GH-V (500 ng/ml) for 30 min. Arteries were again exposed to incremental doses of PHE (10^{-10} – 10^{-5}M) in the presence of GH-V.

There was no significant difference in the increase in active effective pressure induced by each dose of PHE before and after GH-V treatment. Exposure to 500 ng/ml GH-V did not alter the PHE does-response curves or EC80s. Data are summarised in Figure 3.7.
Figure 3.7: GH-V did not alter PHE induced constriction of mice uterine arteries in vitro. PHE dose-response curves were performed pre and post GH-V incubation.

This study demonstrated that human GH-V did not cause attenuation of constriction, or elicit a direct vasodilation effect in uterine arteries of pregnant mice. In vivo, Schiessl et al. observed lower maternal GH-V and IGF-1 levels in association with impaired uterine blood flow in pathologic pregnancies and speculated that GH-V was associated with uterine arterial resistance (Schiessl et al., 2007). In vitro, human GH-N and PRL induced endothelium-dependent vasodilation in isolated rat aortic rings (Gonzalez et al., 2015) and IGF-1 altered the vessel tone of human myometrial, but not placental, arteries (Corcoran et al., 2012). Maternal GH-N, GH-V and IGF-1 concentrations change dramatically during human pregnancy (Chellakooty et al., 2004). Further studies, in terms of species difference and GH-V treatment regimens, are imperative to determine the effect of GH-V on the vessel tone.

3.4.3. The effect of GH-V treatment during mouse pregnancy

To determine the effect of maternal GH-V administration on maternal and fetal growth and metabolic outcomes during pregnancy, the dose response relationship for GH-V
administration in a mouse model of normal pregnancy was examined. Pregnant C57BL/6J mice were randomized to receive vehicle or GH-V (0.25, 1, 2, 5 mg/kg per day) by osmotic pump from gestational days 12.5-18.5.

Maternal body weight and food intake
There was no statistically significant difference in maternal body weight at the time of mating or before osmotic pump implantation. Maternal body weight increased markedly with increasing gestational age in all groups (Figure 3.8A). However, there was no statistically significant difference in maternal body weight and food intake between the vehicle control and GH-V treatment groups (Figure 3.8A and B). A transient reduction in maternal food intake was seen in each group following osmotic pump implantation (Figure 3.8B).
Figure 3.8: Maternal body weight and food intake. A. The change in maternal body weight from GD 0.5 to GD 18.5. B. The change in maternal caloric intake per day from GD 10.5 to 17.5. Data are presented as mean ± SEM. n=6 (0.25, 1, 2 mg/kg/day) or 7 (vehicle, 5mg/kg/day) per group.
Fetal growth and placental weight

There was no statistically significant difference in average litter size in each group (Table 3.1). Pup weight, fetal-abdominal circumference, and placental weight, as well as fetal/placental ratio were not significantly different at GD 18.5 (Figure 3.9A, B and D and Table 3.1). Interestingly, a small decrease in fetal crown-to-rump length was observed in the 5 mg/kg GH-V treatment group, when compared with the vehicle and 0.25 mg/kg treatment groups (29.51 ± 0.15 mm vs 28.73 ± 0.21 mm, p<0.05 and 29.52 ± 0.13 mm vs 28.73 ± 0.21 mm, p<0.05, respectively) (Figure 3.9C). Embryonic mortality was mostly unchanged by GH-V treatment, although an increase in embryo resorption rate (6.56%) was observed in the 5 mg/kg GH-V treatment group (Table 3.1).

Figure 3.9: Pup weight, crown to rump length, abdominal circumference and placental weight. A. Pup weight at GD 18.5. B. Wet placental weight at GD 18.5. C. Pup crown to rump length and D. abdominal circumference at GD 18.5. There were no significant differences in pup weight, fetal abdominal circumference or placental weight following GH-V treatment. However, fetal crown-to-rump length was significantly reduced in the 5 mg/kg GH-V treatment group, compared with the vehicle and 0.25 mg/kg treatment groups. Data are presented as mean ± SEM.
Groups which do not share the same letter are significantly different from each other (p<0.05).

Table 3.1: Fetal and Placental Measurements.

Data are presented as mean ± SEM. N = number of litters per group. There are no significant differences across any of the treatment groups. a:Number of reabsorbed embryos/total number of embryos in each group.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Vehicle (n=7)</th>
<th>0.25mg/kg (n=6)</th>
<th>1mg/kg (n=6)</th>
<th>2mg/kg (n=6)</th>
<th>5mg/kg (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Litter size</td>
<td>8.7 ± 0.4</td>
<td>9.0 ± 0.6</td>
<td>9.2 ± 0.5</td>
<td>8.0 ± 0.5</td>
<td>8.0 ± 0.9</td>
</tr>
<tr>
<td>Pup wt (mg)</td>
<td>1235 ± 26</td>
<td>1188 ± 11</td>
<td>1188 ± 27</td>
<td>1169 ± 26</td>
<td>1165 ± 34</td>
</tr>
<tr>
<td>Placental wt (mg)</td>
<td>85.1 ± 2.2</td>
<td>81.9 ± 1.5</td>
<td>82.2 ± 2.8</td>
<td>82.7 ± 0.7</td>
<td>86.9 ± 3.2</td>
</tr>
<tr>
<td>Fetal-to-placental ratio</td>
<td>14.5 ± 0.5</td>
<td>14.7 ± 0.2</td>
<td>14.3 ± 0.3</td>
<td>13.5 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Reabsorption rate (%)</td>
<td>0</td>
<td>0</td>
<td>1.79</td>
<td>0</td>
<td>6.56</td>
</tr>
</tbody>
</table>

**Maternal tissue weights**

GH-V treatment did not affect the weights of maternal liver, kidneys, spleen or pancreas (Table 3.2). There were no significant differences in maternal adipose tissue weights across all treatment groups; however, a significant dose effect of GH-V on perirenal fat weight was observed (linear, p<0.05; quadratic, p<0.05), with an increase in perirenal fat weight associated with increasing GH-V dose (Figure 3.10A). A similar significant association with dose was observed for gonadal fat weight (linear, not significant; quadratic, p<0.05) (Figure 3.10C). These results suggest that increased GH-V during pregnancy is associated with an increase in maternal adipose deposition.
Table 3.2: Maternal tissue weights and plasma glucose and IGF-1 concentrations.

Data are presented as mean ± SEM. There are no significant differences across any of the treatment groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Vehicle (n=7)</th>
<th>0.25mg/kg (n=6)</th>
<th>1mg/kg (n=6)</th>
<th>2mg/kg (n=6)</th>
<th>5mg/kg (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver wt (mg)</td>
<td>1540 ± 43</td>
<td>1538 ± 34</td>
<td>1565 ± 36</td>
<td>1492 ± 40</td>
<td>1490 ± 68</td>
</tr>
<tr>
<td>Kidney wt (mg)</td>
<td>139.0 ± 4.4</td>
<td>150.9 ± 3.8</td>
<td>145.3 ± 6.1</td>
<td>148.3 ± 6.8</td>
<td>150.6 ± 8.8</td>
</tr>
<tr>
<td>Spleen wt (mg)</td>
<td>124.1 ± 20.8</td>
<td>126.3 ± 14.5</td>
<td>93.3 ± 4.3</td>
<td>97.2 ± 5.3</td>
<td>115.3 ± 10.0</td>
</tr>
<tr>
<td>Pancreas wt (mg)</td>
<td>213.9 ± 12.6</td>
<td>179.8 ± 13.9</td>
<td>210.8 ± 16.9</td>
<td>219.2 ± 8.0</td>
<td>224.3 ± 15.6</td>
</tr>
<tr>
<td>Fed glucose (mmol/L)</td>
<td>14.10 ± 1.0</td>
<td>15.18 ± 0.8</td>
<td>13.40 ± 0.6</td>
<td>14.60 ± 0.5</td>
<td>13.76 ± 1.0</td>
</tr>
<tr>
<td>Fasting glucose (mmol/L)</td>
<td>5.99 ± 0.4</td>
<td>5.70 ± 0.2</td>
<td>6.92 ± 0.5</td>
<td>6.93 ± 0.9</td>
<td>7.16 ± 0.9</td>
</tr>
<tr>
<td>IGF-1 at GD 12.5 (ng/ml)</td>
<td>325.7 ± 21.4</td>
<td>290.4 ± 26.1</td>
<td>280.0 ± 21.0</td>
<td>300.8 ± 17.0</td>
<td>290.7 ± 16.2</td>
</tr>
<tr>
<td>IGF-1 at GD 15.5 (ng/ml)</td>
<td>308.0 ± 10.6</td>
<td>313.8 ± 32.6</td>
<td>308.2 ± 17.5</td>
<td>332.7 ± 17.5</td>
<td>339.6 ± 20.2</td>
</tr>
<tr>
<td>IGF-1 at GD 18.5 (ng/ml)</td>
<td>244.5 ± 33.6</td>
<td>205.1 ± 11.4</td>
<td>281.1 ± 22.4</td>
<td>250.4 ± 31.1</td>
<td>247.5 ± 35.9</td>
</tr>
</tbody>
</table>
Figure 3.10: Maternal adipose tissue weights. A. Perirenal fat. B. Retroperitoneal fat. C. Gonadal fat. There were significant dose effects of GH-V on maternal perirenal and gonadal fat weights using linear and quadratic regression analysis. Bars indicate mean values.
IGF-1, fasting glucose and insulin levels

Maternal IGF-1 increased during mid-pregnancy and decreased in late pregnancy in all treatment groups (Table 3.2). However, GH-V treatment did not affect maternal IGF-1 plasma concentrations at either GD 15.5 or 18.5 (Table 3.2). Maternal fasting insulin levels were significantly increased and insulin sensitivity decreased in the 5 mg/kg treatment group at GD 18.5 (Figure 3.11A and B). A dose-dependent decrease in insulin sensitivity was observed (linear, p<0.01; quadratic, p<0.05) (Figure 3.11C). No affect was seen on fasting glucose levels (Table 3.2).
Figure 3.11: Maternal plasma insulin levels and homeostatic model assessment (HOMA) of insulin sensitivity. A. Maternal fasting insulin levels at GD 18.5. B and C. HOMA for estimation of insulin resistance (IR) and sensitivity (%S) was determined using the HOMA2 Calculator v2.2.3 (Diabetes Trials Unit, University of Oxford). A and B: Data are presented as mean ± SEM. C: Bars indicate mean values. Groups which do not share the same letter are significantly different from each other (p<0.05). There was a dose-dependent decrease in insulin sensitivity using linear and quadratic regression analysis. n=6 (0.25, 1, 2 mg/kg/day) or 7 (vehicle, 5mg/kg/day) per group.
**Hepatic mRNA expression**

The effect of GH-V on hepatic mRNA expression was analysed by comparing gene expression in the vehicle-treated and 5 mg/kg GH-V treatment group (Figure 3.12). Hepatic Ghr/Ghbp and insulin-like growth factor-binding protein 3 (Igfbp3) mRNA levels were significantly increased in the 5 mg/kg treatment group (Vehicle 1.30 ± 0.16 vs GH-V 5mg/kg 1.99 ± 0.11, p<0.01 and 0.92 ± 0.07 vs 1.29 ± 0.12, p<0.05, respectively). Mouse GHBP is generated through alternative splicing of RNA transcripts from the Ghr/Ghbp gene. The primers used in this study do not distinguish between these two transcripts. mRNA levels of the solute carrier family 2, member 4 (Slc2a4, Glut4) were significantly decreased after GH-V treatment (0.92 ± 0.14 vs 0.45 ± 0.08, p<0.05). However, GH-V treatment did not alter the expression of hepatic insulin receptor substrate-1 (Irs-1), insulin receptor (Insr), v-akt murine thymoma viral oncogene homolog 3 (Akt3), Igf-1, IGFBP acid labile subunit (Als), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3-kinase) catalytic subunit alpha (Pik3ca), or PI3-kinase regulatory subunit alpha (Pik3r1).
Figure 3.12: Hepatic mRNA expression. Groups using were compared using Student’s t test. Expression was normalized to the geometric mean of Gapdh, β-Actin and Cox4i1. Bars indicate mean values. *, p<0.05; **, p<0.01

3.5. Discussion

Recombinant GH-N therapy has long been used as an effective treatment for promoting growth due to its somatotrophic properties. However, GH-N treatment can induce insulin resistance, oedema and alterations in carbohydrate and lipid metabolism (Carroll et al., 1998). GH-V is secreted from the placenta during human pregnancy and may
also be associated with fetal growth in humans (Chellakooty et al., 2004; Koutsaki et al., 2011; McIntyre et al., 2000; Mirlesse et al., 1993). Previous studies have observed growth-promoting properties of 22 kDa GH-V in rodents (Barbour et al., 2002; Goodman et al., 1991; MacLeod et al., 1991b; Selden et al., 1988a). However, these studies were conducted in non-pregnant animals. The aim of the current study was to evaluate the physiological effects of GH-V administration in a mouse model of pregnancy.

Despite previous reports of growth-promoting effects in non-pregnant mice, we did not observe any difference in maternal or fetal weight with increasing GH-V dose, although fetal crown-rump length was reduced in the 5 mg/kg treatment group. This is consistent with work by Naar et al. who observed reduced fertility with compromised fetal growth in transgenic mice overexpressing human GH-V (Naar et al., 1991). Other studies have investigated the effect of maternal GH treatment on fetal growth during pregnancy with variable outcomes. Zamenhof et al. treated pregnant rats with bovine GH from day 7 to 20 of pregnancy, with no change in fetal weight but a significant increase in brain weight (Zamenhof et al., 1966). Gargosky et al. treated pregnant rats with recombinant human GH-N or human IGF-1 via an osmotic pump but neither treatment affected fetal or placental weights (Gargosky et al., 1991). In the sheep, Jenkinson et al. treated pregnant ewes with bovine GH during different stages of gestation and found that it stimulated fetal growth only after day 100 of gestation (Jenkinson et al., 1999), while Harding et al. found that neither fetal or maternal growth was altered by bovine GH treatment from day 125 to 134 of gestation (Harding et al., 1997). Discordant results on fetal growth have also been seen in pigs following GH treatment (Gatford et al., 2000; Kelley et al., 1995; Sterle et al., 1995; Tung et al., 2012). It is likely that different GH preparations, dose regimens and treating periods may contribute to these findings.
Moreover, nutrient partitioning may also play an important part in fetal growth (Chiang and Nicoll, 1991; Gatford et al., 2000; Wallace et al., 2004) and it has been suggested that the anabolic effect of exogenous GH on the mother may counteract the growth-promoting effect of GH treatment on the fetus by reducing the nutrient supply (Bauman and Currie, 1980; Harding et al., 1997).

Previous studies have demonstrated that placental lactogen is responsible for the maintenance of pregnancy and a series of actions include promotion of fetal growth in mice (Bartke, 1999; Faria et al., 1991; Markoff and Talamantes, 1981). As human GH exhibits lactogenic activity in rodents (Bartke and Kopchick, 2015), it has been hypothesised that human GH administration may interfere with endogenous lactogen release in the rodent, or that high levels of GH act as an antagonist at the lactogen receptors but exhibit insufficient lactogenic effects during pregnancy (Bartke et al., 1988; Naar et al., 1991). Maternal glucocorticoid levels may also be involved in the effect of GH treatment on fetal growth. Increased maternal glucocorticoid levels impair fetal growth during pregnancy (French et al., 1999; Jobe et al., 1998; Tabor et al., 1991). As elevated glucocorticoid levels are observed in transgenic mice overexpressing the human or bovine GH gene (Cecim et al., 1996; Cecim et al., 1991), maternal glucocorticoids may play a role in fetal growth following GH administration, although the chronic GH effects in transgenic mice may not be comparable to the relatively acute effects of GH administration during pregnancy. In fact, GH administration during pregnancy may elicit a number of interacting effects across the entire neuroendocrine systems. In this study, GH-V treatment did not promote maternal or fetal body weight but impaired fetal linear growth at higher doses. Other possible mechanisms cannot be excluded.
Surprisingly a trend of increased maternal adipose tissue weight with increasing doses of GH-V was observed. Although GH is widely recognised to have lipolytic properties, conflicting reports exist and it has been claimed that GH-N interacts with adipose tissue in different ways to promote both lipolytic and anti-lipolytic effects (Davidson, 1987; Goodman, 1981; Goodman et al., 1991). In *in vivo* studies, it has been shown that GH-N administration reduces lipolysis and free fatty acids in both humans and animals (Cheng and Kalant, 1970; Fineberg and Merimee, 1974; Sirek et al., 1977), although this effect is transient and is only observed in the early period after GH-N injections with subsequent lipolytic effects (Goodman and Grichting, 1983; Hart et al., 1984). In addition, in some studies young GH transgenic mice (≤6 months of age) show increases in fat mass, whereas a reduction in adipose mass is observed in older mice (Kopchick et al., 2014). GH-N and GH-V share similar structures and physiological effects. However, whether GH-V has similar actions on adipose tissue is largely unknown, especially during pregnancy, and the exact effects of GH-V on maternal adipose tissue remain unclear.

GH-V treatment significantly increased maternal fasting plasma insulin concentrations and insulin resistance on GD 18.5 at the 5 mg/kg dose, with no corresponding changes in fasting glucose concentrations. Following conditions of GH deprivation, exposure to GH-N elicits short-term insulin-like effects, whereas chronic GH-N leads to well-documented antagonistic effects on insulin action, and consequently insulin resistance (Carter-Su et al., 1996; Xu and Messina, 2009). The GH-N and insulin receptor signalling pathways share several downstream signalling components. Similar to the action of insulin, GH-N induces tyrosyl phosphorylation of IRS proteins, providing binding sites for the regulatory subunit (p85) of PI3-kinase (Carter-Su et al., 1996; Zhu et al., 2001). GH-N activation of PI3-kinase plays an important role in glucose
transport and lipid synthesis (Carter-Su et al., 1996; Zhu et al., 2001). However, chronic GH-N leads to decreased IRS-1 in skeletal muscle, reduced tyrosine phosphorylation of IRS-1, and stimulates serine phosphorylation of IRS-1 which inhibits interaction of IRS-1 with the insulin receptor (Prattali et al., 2005; Xu and Messina, 2009). GH also increases levels of p85 which competes with p85-p110 heterodimers for binding to IRS-1 and negatively regulates PI3-kinase signalling (Dominici et al., 2005). Studies investigating the effect of GH-V on insulin resistance are more limited. However, a series of studies investigating insulin resistance in transgenic mice (Barbour et al., 2004; Barbour et al., 2002; del Rincon et al., 2007), demonstrated that GH-V does not affect phosphorylation of IRS-1 in the skeletal muscle of transgenic mice (Barbour et al., 2004), but causes insulin resistance by specifically increasing the protein expression of the p85 subunit of PI3-kinase in muscle and adipose tissue and subsequently reducing PI3-kinase signalling (Barbour et al., 2004; del Rincon et al., 2007).

In this study, the mRNA expression levels of hepatic Irs, Insr, Akt3, Pik3ca, and Pik3r1 were unaltered after GH-V treatment. However, hepatic expression of the gene for insulin-sensitive glucose transporter 4 (Slc2a4/Glut4) was significantly down-regulated. Reduced expression of Glut4 has been associated with insulin resistance and plays a role in the pathophysiology of type 2 diabetes mellitus (Kopchick and Andry, 2000). This may contribute to the insulin resistance induced by GH-V. Altered mRNA or protein expression of Irs, Insr, Akt3, Pik3ca, Pik3r1 may occur in other tissues and is under investigation.

IGF-1 is a primary mediator of the effects of GH, in particular its growth-promoting effects. Circulating IGF-1 is synthesized mainly by the liver under the control of GH. The binding of GH with its hepatic receptor stimulates expression and release of IGF-1
into the circulation (Kopchick and Andry, 2000). During human pregnancy, GH-V is secreted continuously from the placenta into maternal circulation from early pregnancy and rises exponentially until 37 weeks of gestation. Maternal circulating IGF-1 concentrations increase dramatically in the second half of pregnancy with a peak at 37 weeks. Concomitantly, maternal GH-N falls to nearly undetectable levels (Verhaeghe, 2008). It is thought that GH-V substitutes for GH-N to regulate maternal circulating IGF-1 concentrations during pregnancy (Caufriez et al., 1993; Handwerger and Freemark, 2000). However, conflicting results exist as to the relationship between maternal IGF-1 concentrations and fetal growth during pregnancy (Boyne et al., 2003; Holmes et al., 1997; Wiznitzer et al., 1998).

Consistent with previous reports (Sheppard and Bala, 1986), we observed that maternal IGF-1 increased during mid-pregnancy and decreased in late pregnancy in mice. However, GH-V treatment did not affect maternal IGF-1 plasma concentrations at either GD 15.5 or 18.5. The effect of exogenous GH administration on IGF-1 levels has been investigated in previous experimental models. Treatment of rats with porcine, bovine GH or human GH-N does increase maternal circulating IGF-1 in some studies (Azain et al., 1995; Cramer et al., 1992; Vickers et al., 2009; Woodall et al., 1999). Others saw no increase in IGF-1 following recombinant human GH-N treatment (Cittadini et al., 1996; Clark et al., 1996), including a study conducted in pregnant rats (Gargosky et al., 1991). Similarly, no increase in IGF-1 was observed in young male Wistar rats treated with the 20 kDa isoform of GH-V (Vickers et al., 2009). However, transgenic mice which overexpress human GH-V have increased IGF-1 both in non-pregnant animals and during pregnancy (Barbour et al., 2002; Naar et al., 1991).

Recent studies suggest that IGF-1 may not be a good biomarker for GH administration in the normal mouse, despite human GH showing bioactivity in rodents. Bielohuby et
al. compared IGF-1 responsiveness in different mouse strains following mouse, bovine and human pituitary GH administration and found no significant increase in IGF-1. However, GH was found to be active, increasing body weight and organ size, and activating STAT5 in the liver. The authors speculate that IGF-1 release is already maximal in these animals and cannot be further increased by exogenous GH treatment (Bielohuby et al., 2011).

The majority of circulating IGF-1 in humans and mice is bound to IGFBP3 and ALS to form a 150 kDa ternary complex and is the major storage form of IGF-1 in the circulation (Baxter, 1988). This complex prolongs the half-life of circulating IGF-1 and facilitates its endocrine actions. IGFBP3 has been shown to either inhibit or augment IGF-1 actions. Overexpressing IGFBP3 in mice resulted in intrauterine fetal growth retardation despite elevated circulating IGF-1 concentrations (Modric et al., 2001). On the other hand, both IGF-1 and IGFBP3 are elevated concordantly in acromegaly patients and yet there is no growth inhibition due to the equilibrium between IGF-1 and IGFBP3 concentrations (Grinspoon et al., 1995). The liver is believed to be the main source of IGF-1, IGFBP3, and ALS in the circulation (Le Roith et al., 2001). Hepatic Igfbp3 mRNA levels were significant increased after GH-V treatment. In addition, there was a trend for increased Als mRNA expression while maternal plasma IGF-1 concentrations were unaltered. Increased IGFBP3 may diminish free IGF-1 in the maternal circulation and thus reduce the bioactivity of IGF-1. This may partially explain the reduced fetal length observed in this study.

GH-V increased liver Ghr/Ghbp expression during mouse pregnancy. This is consistent with studies using GH in pregnant mice (Cramer et al., 1992) and rats (Maiter et al., 1988). Jiang et al. found that GH increased hepatic Ghr expression in a bovine model (Jiang et al., 2007). However, Mathews et al. found no significant changes in hepatic
Ghr mRNA levels between control and hypophysectomised rats treated with bovine GH, although pregnant females had elevated Ghr expression (Mathews et al., 1989). The time, dose and duration of GH exposure, in vivo or in vitro experiments, steroid hormones, and nutritional status may all contribute to the variation shown across models (Schwartzbauer and Menon, 1998).

In contrast to the situation in humans, mouse GHBP is generated through alternative splicing of RNA transcripts from the Ghr/Ghbp gene (Talamantes and Ortiz, 2002). Circulating concentrations of pituitary GH increase during mouse pregnancy. Expression of the mouse GHR and GHBP also increase substantially. It is therefore thought that the concentration of free circulating GH may remain unchanged during mouse pregnancy (Cramer et al., 1992; Talamantes and Ortiz, 2002). Early studies demonstrated that mouse liver GHBP predominantly exists as a membrane-associated protein which is structurally distinct from the soluble form of GHBP present in serum. It has been suggested that membrane-associated GHBP may function as a cell-surface receptor for GH in the liver through interaction with integrins (Cerio et al., 2002; Talamantes and Ortiz, 2002). However, an alternative explanation is that membrane associated GHBP may act to attenuate the effects of high concentrations of GH (Gonzalez et al., 2007).

There are other differences in the GH axis in mouse and human pregnancy which should be highlighted with regards to this study. As described above, the human GH gene family is a cluster of five genes, which includes GH-N, GH-V, and the chorionic somatomammotropin (CS-A, CS-B and CS-L) genes (Fiddes et al., 1979). In contrast, the rodent genome contains a single pituitary-specific GH gene and lacks any GH-related CS genes (Hall and Talamantes, 1984). Consequently, only a pituitary version of GH is expressed during rodent pregnancy (Cramer et al., 1992; Talamantes and
Ortiz, 2002). It is not clear why the primate locus has evolved independently to encompass a pituitary and placental version of the GH gene. Despite these differences, extra-pituitary expression of GH is observed in multiple tissues in both humans and mice, including the mouse placenta (Harvey et al., 2000a), which suggests a potential role in mouse pregnancy. So although the mice lack a GH-V gene, the mouse is still a useful model to determine effects of GH-V. Further studies would be required to determine whether there are differences in the action of GH-N versus GH-V, or whether the mode of presentation of GH (pulsatile versus tonic) may be more important.

Although human GH-N and GH-V can both bind and activate the GHR of non-primate species, species-specific differences cannot be excluded.

In conclusion, higher doses of GH-V induced hyperinsulinemia in pregnant C57BL/6J mice, suggesting that GH-V is a likely candidate to induce insulin resistance during pregnancy. Although GH-V treatment did not promote fetal growth in this studies, due to the intimate relationship between GH-V and fetal growth during human pregnancies, further investigation of specific animal models, particularly those of growth restriction, are warranted.
Chapter 4: Comparison of pulsatile versus continuous administration of placental growth hormone in mice

4.1. Preface

The following chapter contains data from a published article “Comparison of pulsatile versus continuous administration of human placental growth hormone in female C57BL/6J mice” (Appendix II). A version of this chapter was published in Endocrine in 2016. Endocrine is a peer-reviewed medical journal covering endocrinology. The journal had an impact factor of 3.878 in 2014.

GH-N exhibits different growth-promoting properties in different administration methods (Jansson et al., 1982a). In humans, GH-N is secreted from the pituitary in a pulsatile manner, whereas GH-V is expressed continuously from the placenta (Eriksson et al., 1989). In order to determine the effect of GH-V via different administration methods, the effect of pulsatile versus continuous administration of recombinant GH-V was investigated in a normal mouse model.

4.2. Introduction

The effects of exogenous GH-N on growth, body composition and carbohydrate metabolism are well documented and it has been noted that different effects are observed in response to different modes of delivery. Pulsatile infusion of human or bovine GH, compared to continuous infusion, is more effective in stimulating growth in hypophysectomized rats (Clark et al., 1985; Isgaard et al., 1988). Differences in growth rate and body composition were also observed in intact rats (Azain et al., 1993; Azain et al., 1995). In addition, GH-N has different effects on glucose homeostasis and lipid
profiles in GH-deficient adults depending on whether administration is by daily injection or continuous infusion (Oscarsson et al., 1996). Although GH-V has been demonstrated to stimulate growth, alter body composition and induce insulin resistance in hypophysectomized rats and transgenic mice (Barbour et al., 2002; MacLeod et al., 1991b; Selden et al., 1988b), there is relatively little information on the effects of GH-V utilising different modes of administration.

The aim of the current study was to determine the effect of delivery methods on growth and metabolic outcomes by examining the effect of pulsatile versus continuous administration of recombinant GH-V in the non-pregnant female mouse.

4.3. Hypothesis and aims

The hypothesis is that different modes of delivery of GH-V administration have distinct impacts on growth and metabolic outcomes in mice.

The aims of this chapter are therefore:

a. To determine the bioactivity of GH-V in osmotic pumps over the treatment period;

b. To determine the effect of GH-V on Igf-1 mRNA expression in a mouse cell line;

c. To determine the effect of GH-V treatment via pulsatile versus continuous administration on growth and metabolic outcomes, including body weight, body composition, and insulin sensitivity;

d. To elucidate the underlying mechanisms on how modes of delivery of GH-V impact on growth and metabolism.

4.4. Recombinant GH-V protein stability

The bioactivity of GH-V in osmotic pumps over the treatment period was estimated by incubating GH-V at 37°C for 6 days and measuring the phosphorylation of STAT5 in
GH-V-treated BA/F3-GHR cells using an AlphaScreen assay. A 26% reduction in GH-V bioactivity was observed after 6 days incubation at 37°C (Figure 4.1). No statistically significant difference in bioactivity was observed from day 0-3. Dose response curves were carried out on day 0, 3 and 6 and the EC50 measured. A small increase in the EC50 concentration was observed at day 3 (152.2 ng/ml) and day 6 (122.8 ng/ml), compared to day 0 (90.9 ng/ml), however, these differences were not significant.

Figure 4.1: GH-V stability following incubation at 37°C. GH-V bioactivity was determined by measuring phosphorylation of STAT5 in BAF3-GHR cells using an AlphaScreen assay. Cells were treated with 500 ng/ml GH-V for 10 min. Data are presented as mean ± SEM of duplicate measurements.

4.5. Effect of pulsatile versus continuous administration of GH-V treatment on mice

A total of 36 mice, averaging 19.5 ± 0.1g initial body weight, were randomized to receive GH-V (2 or 5 mg/kg per day; calculated on the basis of pre-treatment body weight) or vehicle for six days by either subcutaneous injection (SC) or osmotic pump (OP) (Alzet model 1007D, Durect Corporation, Cupertino, CA) (n=6 for each group).
For SC groups, GH-V or vehicle (100 µl) was administered subcutaneously in the skinfold at the nape of the neck using tuberculin syringes twice a day (8am and 5pm) from day 1 to day 6 with the last injection occurring on day 7 at 8am. Lyophilized powder was solubilized to the target concentration prior to use on each treatment day.

For OP groups, pump selection was based on the size, duration and flow rate. Model 1007D (100 µl) was designed to release its contents at a rate of 0.5 µl/h over one week duration. Pumps were filled with reconstituted recombinant human GH-V or vehicle and placed in sterile 0.9% saline at 37°C for priming overnight. On day 1, pumps were inserted on the back of animal, slightly posterior to the scapulae. On day 4, a blood sample was obtained via tail tip from the OP groups. Delivery was verified by measurement of the residual volume in the pump reservoir after removal on day 7.

Body weights and food intake were monitored daily from day 0 to day 7. On day 7, mice were fasted for 4h from 10am to 2pm, and euthanized by cervical dislocation. Blood was collected by cardiac puncture and kept on ice until centrifugation and removal of supernatant for plasma analysis. Glucose measurements were performed with a Freestyle Optimum glucometer (Abbott, UK). The weights of the liver, kidneys, spleen, heart, perirenal fat, retroperitoneal fat and gonadal fat were recorded and tissues stored at -80°C.

4.5.1. Body weight and food intake

GH-V treatment with 2 and 5 mg/kg per day via SC significantly increased body weight (repeated measures ANOVA, p<0.05) without affecting food intake, compared with vehicle treated animals (Figure 4.4 A-D). There was no statistically significant difference in body weight or food intake among groups following OP treatment (Figure
4.4 C-D), although a transient reduction in maternal food intake was seen following osmotic pump implantation (Figure 4.4 D).

Figure 4.2: Body weight and food intake. Data are presented as mean ± SEM. Groups which do not share the same letter are significantly different from each other (p < 0.05).

4.5.2. Tissue weights

GH-V treatment with 5 mg/kg per day via SC significantly increased liver, kidney and spleen weights (one-way ANOVA, p < 0.05), but only liver weight was increased following 2 mg/kg per day SC treatment (one-way ANOVA, p < 0.01) (Figure 4.5). GH-V treatment via OP did not affect the weight of the liver, kidneys, or spleen (Fig. 3). Heart weight was not affected by different administration methods or treatment doses. There were no significant differences in adipose tissue weights across treatment groups (Figure 4.6).
Figure 4.3: Tissue weights. Bars indicate mean values. Groups which do not share the same letter are significantly different from each other (p < 0.05).
4.5.3. Plasma IGF-1, fasting glucose and insulin concentrations

In the SC groups, there was no effect of GH-V treatment on IGF-1 plasma concentration after 6 days of treatment (Figure 4.7A). In the OP treatment group, GH-V treatment did not affect IGF-1 plasma concentration at day 4 (Figure 4.7B). However, a significant dose effect of GH-V on IGF-1 concentration was observed (linear trend,
p<0.05), with a decrease in IGF-1 concentration associated with increasing GH-V dose at day 7 in OP groups (Figure 4.7C).

Figure 4.5: Plasma IGF-1 concentrations. Bars indicate mean values.

As no stimulatory effect on circulating IGF-1 was observed, I tested whether GH-V was capable of stimulating Igf-1 mRNA expression in the mouse myoblast cell line C2C12, which is known to up-regulate Igf-1 mRNA expression in response to GH-N treatment (Frost et al., 2002; Imanaka et al., 2008). Both GH-N and GH-V increased expression of Igf-1 in this cell line.

Fasting insulin concentrations were significantly increased in the 5 mg/kg SC treatment group, compared to vehicle (one-way ANOVA, p<0.05) (Figure 4.8A). However, fasting insulin concentrations were not significantly different from the vehicle treated
control in the OP groups (Figure 4.8B), a dose-dependent increase in fasting insulin concentration and decrease in insulin sensitivity was observed in both SC and OP administration treatment groups, as assessed by HOMA (linear trend, p<0.05) (Figure 4.8A-D). No effect was seen on fasting glucose concentrations.

![Figure 4.6: Plasma fasting insulin concentrations and homeostatic model assessment (HOMA) of insulin resistance. A and B. Fasting insulin concentrations at day 7. C and D. Bars indicate mean values. Groups which do not share the same letter are significantly different from each other (p <0.05). There was a dose-dependent increase in insulin concentrations and HOMA-IR using linear regression analysis (p <0.05).]

4.5.4. Hepatic mRNA expression

The effect of GH-V on hepatic mRNA expression was analysed by comparing gene expression in the vehicle-treated and 5 mg/kg GH-V treatment group (Table 4.1). GH-V treatment via SC and OP significantly increased hepatic Ghr/Ghbp expression...
(p<0.05) (Table 4.1). It should be noted that mouse growth hormone binding protein
(GHBP) is generated through alternative splicing of RNA transcripts from the Ghr
gene. The primers used in this study do not distinguish between these two transcripts.

mRNA expression of hepatic Igf-1 and insulin-like growth factor-binding protein 3
(Igfbp3) was not affected by GH-V treatment, but IGFBP acid labile subunit (Als)
expression was significantly decreased after GH-V OP treatment (p<0.05). Moreover,
mRNA levels of v-akt murine thymoma viral oncogene homolog 1 (Akt1) in SC group
was significantly increased (p<0.05), whereas expression in the OP group was not
affected. Additionally, changes in insulin receptor (Insr) expression after GH-V OP
treatment exhibited a marginal trend toward significance (p=0.051). However, GH-V
treatment did not alter the expression of hepatic insulin receptor substrate-1 (Irs-1),
Akt2, Akt3, Solute carrier family 2, member 1, 2 and 4 (Glut1,2 and 4),
phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3-kinase) catalytic subunit alpha
(Pik3ca), or PI3-kinase regulatory subunit alpha (Pik3r1).
### Table 4.1. Hepatic mRNA expression.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>SC Group Fold Change</th>
<th>SC Group p-value</th>
<th>OP Group Fold Change</th>
<th>OP Group p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ghr</td>
<td>1.71</td>
<td>0.010</td>
<td>1.66</td>
<td>0.003</td>
</tr>
<tr>
<td>Insr</td>
<td>-1.30</td>
<td>0.051</td>
<td>1.08</td>
<td>0.626</td>
</tr>
<tr>
<td>Irs-1</td>
<td>-1.14</td>
<td>0.491</td>
<td>1.30</td>
<td>0.071</td>
</tr>
<tr>
<td>Akt1</td>
<td>1.25</td>
<td>0.029</td>
<td>-1.00</td>
<td>0.988</td>
</tr>
<tr>
<td>Akt2</td>
<td>-1.09</td>
<td>0.716</td>
<td>1.30</td>
<td>0.192</td>
</tr>
<tr>
<td>Akt3</td>
<td>-1.22</td>
<td>0.212</td>
<td>1.16</td>
<td>0.329</td>
</tr>
<tr>
<td>Pik3ca</td>
<td>1.19</td>
<td>0.253</td>
<td>1.28</td>
<td>0.218</td>
</tr>
<tr>
<td>Pik3r1</td>
<td>1.10</td>
<td>0.466</td>
<td>1.21</td>
<td>0.670</td>
</tr>
<tr>
<td>Glut1</td>
<td>-1.19</td>
<td>0.217</td>
<td>1.01</td>
<td>0.932</td>
</tr>
<tr>
<td>Glut2</td>
<td>-1.05</td>
<td>0.724</td>
<td>-1.08</td>
<td>0.560</td>
</tr>
<tr>
<td>Glut4</td>
<td>-1.52</td>
<td>0.237</td>
<td>-1.33</td>
<td>0.066</td>
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<tr>
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<td>0.378</td>
<td>-1.04</td>
<td>0.656</td>
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<tr>
<td>Igfbp3</td>
<td>1.11</td>
<td>0.320</td>
<td>-1.06</td>
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</tr>
<tr>
<td>Als</td>
<td>1.56</td>
<td>0.071</td>
<td>-1.41</td>
<td>0.020</td>
</tr>
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</table>

Control group and 5 mg/kg GH-V treatment group were compared using Student’s t test. Expression was normalized to the geometric mean of Gapdh, β-Actin and Cox4i1. Positive and negative fold changes indicate a respective increase or decrease in mRNA levels.

### 4.6. Discussion

Previous studies have found that the induction of the growth and metabolic response to GH varies according to the dose administered and the delivery method used (Clark et al., 1985; Laursen et al., 1996; Roelfsema et al., 2001); however studies with GH-V had not been conducted. In the present study, the effects of pulsatile versus continuous administration of recombinant GH-V was compared, in terms of growth and metabolic outcomes in female C57BL/6J mice. GH-N secretion is highly episodic in mammals. This intermittent secretion is of importance for the biological effects in peripheral tissues. A sexually dimorphic GH-N secretory pattern has been observed in rodents and
humans and regulates the expression of several sex-specific phenotypes (Eden, 1979; Jaffe et al., 1998; Jansson et al., 1985; MacLeod et al., 1991a). In females, the pulses of GH-N secretion are lower and plasma GH-N baseline level is higher than in males (MacLeod et al., 1991a).

The effects of different modes of GH-N delivery on regulation of growth have been studied previously. Jansson et al. (Jansson et al., 1982b) and Thorngren et al. (Thorngren and Hansson, 1977) observed that the frequency of GH administration influenced body growth in hypophysectomized rats. However, the growth response did not increase proportionally to an increased administration frequency. The "stress" associated with injections may contribute to this. To avoid frequent injections, an osmotic pump was designed which allowed continuous administration. In terms of growth stimulation, pulsatile GH administration has been inferred to be superior to continuous delivery (Maiter et al., 1988). Clark et al. treated hypophysectomised rats with recombinant human growth hormone (0.04, 0.2, 1, or 5 mg/kg per day) for 7 days and found that growth responses depended on the pattern of GH administration (twice daily injections > continuous infusions > daily injections) (Clark et al., 1995).

Consistent with previous studies, GH-V treatment via twice daily SC was more effective in stimulating growth than OP and significantly increased body weight and organ size. However, despite administration in comparable doses, GH-V treatment via OP did not affect animal growth.

The mechanism underlying this differential responsiveness to the mode of administration is still unclear. IGF-1 is a primary mediator of the effects of GH-N, in particular its growth-promoting effects. Circulating IGF-1 is synthesized mainly by the liver under the control of GH-N. The binding of GH with its hepatic receptor stimulates expression and release of IGF-1 into the circulation (Kopchick and Andry, 2000).
Moreover, it has been proposed that GH-V substitutes for GH-N to regulate maternal circulating IGF-1 concentration during pregnancy (Caufriez et al., 1993; Handwerger and Freemark, 2000). Transgenic mice which overexpress human GH-V have increased circulating IGF-1 concentration (Barbour et al., 2002; Naar et al., 1991). Maiter et al. found that GH-N administration with intermittent delivery (4 times a day) caused higher serum IGF-1 concentration that with continuous delivery in rats (Maiter et al., 1988). In this study, GH-V treatment did not affect plasma IGF-1 concentration or hepatic Igf-1 expression via either SC or OP administration but a trend for decreasing IGF-1 concentrations was observed with increasing GH-V doses in the OP group. Of note, a recent study suggests that IGF-1 may not be a good biomarker for GH activity in wild-type mice, despite human GH showing bioactivity in rodents. Bielohuby et al. compared IGF-1 responsiveness in different mouse strains following mouse, bovine and human GH-N administration and found no significant increase in IGF-1. The authors speculate that IGF-1 release is already maximal in normal animals and cannot be further increased by exogenous GH treatment (Bielohuby et al., 2011). Nonetheless this does not exclude the possibility that local generation of IGF-1 in some tissues apart from liver may play a role in the growth promoting effect of GH. Isgaard et al. observed that pulsatile and continuous GH treatment were equally effective in stimulating hepatic Igf-1 expression in hypophysectomized rats, whereas only pulsatile GH infusion had a marked stimulatory effect in skeletal muscle (Isgaard et al., 1988). Further, different patterns of GH treatment also affect GH sensitivity by altering GH binding. Continuous infusion of GH has been found to be more potent in increasing the number of GH and prolactin binding sites than injections (Bick et al., 1992; Maiter et al., 1988). Baumbach et al. found that GH treatment by continuous infusion significantly induced Ghr expression in hypophysectomised female and castrated male
rats, but a single injection did not (Baumbach and Bingham, 1995). These findings are consistent with reports of hepatic GHR and serum GHBP in dwarf rats (Carmignac et al., 1993; Carmignac et al., 1992). Additionally, only a small percentage of the mouse GH is bound to GHBP in normal mice. Given that the binding affinity of human GH for the murine GHR and GHBP is higher than the affinity of mouse GH (Gonzalez et al., 1999), different circulating GH-V concentrations generated by administration patterns may have different impacts on GH-GHBP binding and effects in mice.

In the present study it was found that GH-V treatment utilising two modes of administration significantly increased hepatic Ghr/Ghbp expression in mice. In contrast to humans, the mouse GHR gene contains a special exon (exon 8A) which encodes a GHBP specific hydrophilic sequence (Edens et al., 1994). Therefore, GHBP is generated through alternative splicing of RNA transcripts from the Ghr/Ghbp gene (Talamantes and Ortiz, 2002) and its expression clearly depends on GH concentrations (Gonzalez et al., 1999; Sanchez-Jimenez et al., 1990). Early studies demonstrated that mouse liver GHBP predominantly exists as a membrane-associated protein which is structurally distinct from the soluble form of GHBP present in serum, and the membrane-associated GHBP may function as a cell-surface receptor for GH in the liver through interaction with integrins (Cerio et al., 2002; Talamantes and Ortiz, 2002). However, an alternative explanation is that membrane associated GHBP may act to attenuate the effects of high concentrations of GH (Gonzalez et al., 2007).

The majority of circulating IGF-1 in humans and mice is bound to IGFBP3 and ALS to form a 150 kDa ternary complex and is the major storage form of IGF-1 in the circulation (Baxter, 1988). ALS stabilizes the IGF-IGFBP3 complex, reduces the passage of IGF-1 to the extravascular compartment, prolongs the half-life of circulating IGF-1 and facilitates endocrine actions of IGF-1. GH is believed to be the most potent
inducer of ALS mRNA in the liver (Ooi et al., 1997). The effects of GH on the Als gene are mediated by the JAK-STAT pathway (Carter-Su et al., 1996); both GH-N and GH-V have been previously showed to activate this pathway (Kopchick and Andry, 2000; Lacroix et al., 2002a). In the SC group there was a trend for increased Als expression; however this was not significant. Surprisingly, continuous treatment of GH-V (5 mg/kg per day) induced a slight decrease in hepatic Als mRNA expression (1.4-fold). A decrease in Als expression may contribute to the dose-dependent decrease in IGF-1 concentration observed in OP groups, as previous studies have found that ALS depletion induces a mild growth deficit despite a large reduction in the concentration of IGF-1 (Domene et al., 2004; Ueki et al., 2000).

While GH is widely recognised to be lipolytic, conflicting reports exist and it has been claimed that GH-N interacts with adipose tissue in different ways to promote both lipolytic and anti-lipolytic effects (Davidson, 1987; Goodman, 1981). Moreover, free fatty acid concentrations increased after GH treatment in both normal individuals (Metcalf & et al., 1981) and GH deficient patients (Salomon et al., 1989). However, the lipolytic effect of GH-N is more pronounced via pulsatile administration (Johansson et al., 1996; Laursen et al., 1995). Although GH-V exhibits similar effects to GH-N on fat metabolism in the rat (Goodman et al., 1991), adipose tissue weight was not altered by GH-V treatment in this study. The exact effects of GH-V on adipose tissue remain unclear.

GH is an important regulator of insulin sensitivity. Following conditions of GH deprivation, exposure to GH-N elicits short-term insulin-like effects, whereas chronic GH-N leads to well-documented antagonistic effects on insulin action, and consequently insulin resistance (Xu and Messina, 2009). The GHR and insulin receptor signalling pathways share several downstream signalling components (Carter-Su et al.,
Similar to the action of insulin, GH-N induces tyrosyl phosphorylation of IRS proteins, providing binding sites for the regulatory subunit (p85) of phosphoinositide 3-kinase (PI3-kinase) (Dominici and Turyn, 2002). GH-N activation of PI3-kinase and its downstream effector plays an important role in glucose transport and lipid synthesis (Carter-Su et al., 1996; Costoya et al., 1999). However, chronic excess GH-N exposure is associated with decreased response to insulin stimulation by decreasing IRS-1 in skeletal muscle, reducing tyrosine phosphorylation of IRS-1, and stimulating serine phosphorylation of IRS-1 which inhibits interaction of IRS-1 with the insulin receptor (Prattali et al., 2005; Xu and Messina, 2009). GH also increases levels of p85 which competes with PI3-kinase heterodimers for binding to IRS-1 and negatively regulates PI3-kinase signalling (Dominici et al., 2005). Administration pattern of GH has been shown to influence insulin sensitivity. Higher insulin concentration was seen in continuous GH treatment in rats (Azain et al., 1995). In GH deficient patients, daily GH injections administered for a short period (2-4 weeks) exacerbated impaired glucose tolerance when compared to continuous infusion (Johansson et al., 1996; Laursen et al., 1995), but the GH pattern did not influence insulin sensitivity after 6 months treatment (Laursen et al., 2001). Studies investigating the effect of GH-V on insulin resistance are more limited. A series of studies investigating insulin resistance in transgenic mice (Barbour et al., 2004; Barbour et al., 2002; del Rincon et al., 2007), demonstrated that GH-V does not affect phosphorylation of IRS-1 in the skeletal muscle of transgenic mice (Barbour et al., 2004), but causes insulin resistance by specifically increasing the protein expression of the p85 subunit of PI3-kinase in muscle and adipose tissue and subsequently reducing PI3-kinase signalling (Barbour et al., 2004; Caufriez et al., 1993; del Rincon et al., 2007).
It was observed that GH-V treatment via SC significantly increased fasting plasma insulin concentrations at the 5 mg/kg dose, with no corresponding changes in fasting glucose concentrations. A dose-dependent increase in fasting insulin concentration and insulin resistance was also observed in all treatment groups via SC and OP. The mRNA expression levels of components of the insulin signalling pathway in the liver was investigated and it was found that hepatic \textit{Irs, Insr, Akt2, Akt3, Pik3ca, Pik3r1, Glut1, Glut2} and \textit{Glut4} expression were not significantly different to vehicle after GH-V treatment via either delivery modes but \textit{Akt1} expression in the SC group was significantly increased.

AKT is a down-stream target of PI3-kinase signalling and mediates most of the PI3-kinase mediated metabolic actions, including the translocation of GLUT4. In mammals, AKT has three isoforms, encoded by different genes (\textit{Akt1-3}). These isoforms have some differential and nonredundant physiological functions, suggested by phenotypes of mice lacking individual AKT isoforms. AKT1 is essential for cell survival and body growth, but dispensable for maintenance of glucose homeostasis in mice (Cho \textit{et al.}, 2001b). In contrast, AKT2 deficient mice display insulin resistance and diabetes phenotype, contributed by the inability of insulin to induce glucose utilization and decrease hepatic glucose output (Cho \textit{et al.}, 2001a). AKT3, however, has no proven metabolic effects.

Previously we tested GH-V in normal pregnant mice and found that hepatic expression of \textit{Glut4} gene was significantly down-regulated with concomitant hyperinsulinemia (Liao \textit{et al.}, 2016b). A trend for decreased \textit{Glut4} expression was also seen in the present study. The insulin resistance induced by GH-V may result from reduced expression of \textit{Glut4}, which has been associated with insulin resistance (Abel \textit{et al.}, 2001; Kopchick and Andry, 2000). Altered mRNA or protein expression may occur in
other insulin target tissues, including muscle (Garvey et al., 1998; Zisman et al., 2000) and adipose tissue (Guilherme et al., 2008; Shepherd and Kahn, 1999), and is currently under investigation.

The secretion of GH from the pituitary is controlled by a number of neuronal, hormonal and metabolic factors. In addition, GH and IGF-1 have a feedback effect, either alone or in combination, to inhibit GH secretion (Clark et al., 1988). It is commonly believed that exogenous GH treatment suppresses endogenous GH secretion, although the mechanism is unclear, as are the sites of action. It is therefore possible that different delivery modes of exogenous GH-V impacted differently on the secretion of endogenous GH, and may have contributed to the different responses between pulsatile and continuous treatments observed. In order to clarify this, further studies in GH-deficient models are warranted, to control for the effects from endogenous GH.

This is the first study to investigate the effects of different modes of GH-V treatment in mice. In conclusion, pulsatile and continuous administration of GH-V had similar effects on the variables described; pulsatile treatment was more effective in stimulating growth but caused marked hyperinsulinemia. Independently from the mode of GH administration, the time, dose and duration of GH exposure, the sex and strains of animals may all contribute to those variations observed in previous studies. Additional studies are necessary to evaluate the differential effects between GH-V and GH-N.
Chapter 5: Optimisation of a GH-V ELISA and determination of the serum concentrations of GH-V and related proteins in pathological human pregnancies

5.1. Preface

Previous studies had demonstrated an associated between GH-V and a number of pathological pregnancy conditions, including FGR, GDM, PE and other conditions (detailed in Chapter 1). In this chapter, an in-house GH-V assay was developed due to the lack of a sensitive and specific commercially available assay. Using this assay, the relationship between GH-V, as well as IGF-1, IGF-2, IGFBP-1 and IGFBP-3, and pregnancies complicated by inappropriate fetal growth, GDM and PE were investigated.

Section 5.3. contains data from a peer-reviewed article “Human placental growth hormone is increased in maternal serum at 20 weeks of gestation in pregnancies with large-for-gestational-age babies”. A version of this section was published in Growth Factors in 2017.

Sections 5.4. and 5.5. contain data from two original research articles “Maternal serum concentrations at 20 weeks’ gestation of IGF-1 and IGFBP-1, but not placental growth hormone, are associated with pregnancies complicated by gestational diabetes mellitus” and “Maternal serum IGF-1, IGFBP-1 and 3, and placental growth hormone at 20 weeks’ gestation in pregnancies complicated by preeclampsia”, which were submitted to Hormones and Pregnancy Hypertension, respectively (under review).
5.2. The development of a GH-V ELISA

5.2.1. Introduction

The ELISA is a powerful method for detecting and quantifying a specific protein in a complex mixture. ELISA typically requires that the antigen of interest be captured or immobilized on a solid surface and then be complexed with an antibody that is linked to an enzyme. Detection is accomplished by assessing the conjugated enzyme’s activity via incubation with a substrate to produce a measurable product. The technique has revolutionized immunology and is commonly used in medical research laboratories. Many ELISAs are available commercially.

ELISA can be performed in different formats (direct, indirect, or sandwich), depending on the same basic elements: capture, blocking, probing and signal measurement. The key step, immobilization of the antigen of interest, can be accomplished by direct adsorption to the assay plate or indirectly via a capture antibody (sandwich) that has been attached to the plate. The antigen is then detected either directly (labelled primary antibody) or indirectly (labelled secondary antibody). The most powerful ELISA assay format is the sandwich assay because it is sensitive and robust.

GH was firstly detected in maternal blood with the use of two monoclonal antibodies (K24 and 5B4) (Eriksson et al., 1988; Hennen et al., 1985). 5B4 reacts with the N-terminal epitope and recognizes both GH-N and GH-V; K24 reacts with an internal epitope and exclusively recognizes GH-N. The concentration of GH-V was calculated from the difference in results obtained from two radio immunoassays (RIA) with these antibodies (Evain-Brion et al., 1994). With the use of purified recombinant human GH-V, G. Hennen's laboratory produced two monoclonal antibodies E8 and 7C12, which specifically recognized GH-V (Igout et al., 1993). By using E8 and 7C12 in a $^{125}$I-
labbeled sandwich immunoassay, maternal levels of GH-V were able to be directly measured (McIntyre et al., 2000). RIA has very high sensitivity and specificity. However, the main drawbacks to RIA are the expense and hazards of using radioactive reagents, the sample volume required and it requires appropriate counting and monitoring devices. With comparative sensitivity and ease of handling, the sandwich ELISA is a useful tool for analyte measurement and requires lower volume of the samples.

Several commercial GH-V ELISAs were tested for activity against recombinant GH-V, GH-N, and serum from pregnant and non-pregnant women. However, these either didn’t detect recombinant GH-V, or GH-V in serum from pregnant females, or they exhibited significant cross-reactivity with GH-N. As the volume of sample available was limited, an in-house ELISA was developed for GH-V analysis. A series of different GH-V and GH-N antibodies were tested for reactivity against GH-V and GH-N using an indirect ELISA. Consistent with previous reports, antibodies E8 and 7C12 were identified as having potential utility. However, as both antibodies were of mouse origin, one antibody of the antibody pair needed to be biotinylated.

5.2.2. Materials and Methods

Recombinant human hormone proteins

Recombinant human GH-V (22 kDa) was purchased from Protein Laboratories Rehovot (Rehovot, Israel) and was reconstituted in 0.4% NaHCO3 pH 9 (Solomon et al., 2006). Recombinant human GH-N (22 kDa) and placental lactogen (PL) were obtained from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA, US).
Human GH-V antibodies

Human GH-V monoclonal antibodies E8 and 7C12 (Bio-Rad AbD Serotec, US) specific recognized GH-V: E8 does not cross react with GH-N or PRL; 7C12 shows some cross reactivity with GH-N (5%) as per details provided by the manufacturer.

Other materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immuno Nonsterile 96-Well Plates</td>
<td>Thermo Fisher Scientific, NZ</td>
</tr>
<tr>
<td>ELISA Ultradextrin</td>
<td>Bio-Rad AbD Serotec, US</td>
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<tr>
<td>LYNX Rapid Biotin (Type 1) Antibody Conjugation Kit</td>
<td>Bio-Rad AbD Serotec, US</td>
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<tr>
<td>Horseradish Peroxidase conjugated Streptavidin (Streptavidin:HRP)</td>
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<tr>
<td>TMB Substrate Reagent Set</td>
<td>BD Biosciences, US</td>
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<tr>
<td>Wash Buffer (PBS-T)</td>
<td>0.05% Tween-20 in PBS, pH 7.4</td>
</tr>
<tr>
<td>Stop Solution</td>
<td>2N H2SO4</td>
</tr>
<tr>
<td>Blocking Buffer / Sample Diluent</td>
<td>2% non-fat milk in PBS</td>
</tr>
<tr>
<td>Coating Buffer</td>
<td>0.1M Sodium Carbonate, pH 9.5</td>
</tr>
</tbody>
</table>

Instrument

The microplate washer and spectrophotometer was obtained from Biotek (Bad Friedrichshall, Germany). Data was analysed using Gen5 Microplate Reader and Imager Software.

5.2.3. ELISA procedure

Indirect ELISA procedure

Microwell plates were coated with antigen diluted in phosphate buffer by overnight incubation at 4°C. The coated plate was washed three times with washing buffer and 200 µl blocking buffer was added to each well followed by 1 h incubation at room temperature. After this the plate was washed three times with washing buffer. 100 µl primary antibody was added to each well and incubated at room temperature for 2 h. The plate was washed three times and 100 µl conjugated secondary antibody was added.
followed by 1 h incubation at room temperature. Following this the plate was washed four times with washing buffer. 100 µl substrate solution was added to each well and incubated at room temperature for 20 min in the dark. Finally, 50 µl stop solution was added to each well. Absorbance was read at 450nm and 590 nm within 30 min of stopping the reaction.

**Sandwich ELISA procedure**

Microtiter plates were coated with antibody diluted in phosphate buffer by overnight incubation at 4°C. The coated plate was washed three times with washing buffer. Blocking was achieved by 1 hour incubation at room temperature with 200 µl blocking buffer. 100 µl sample or standards was added to each well and incubated at room temperature for 2 h. The plate was washed three times with washing buffer and 100 µl biotinylated detection antibody was added to each well followed by 1 h incubation at room temperature. The plate was washed again three times with washing buffer. 100 µl enzyme conjugated streptavidin was added to each well followed by 30 min incubation at room temperature. Following this, the plate was washed four times. 100 µl substrate solution was added to each well and incubated at room temperature for 20 min in the dark. Finally 50 µl stop solution was added to each well. Absorbance was read at 450 nm and 590 nm within 30 min of stopping reaction.

**Biotin antibody conjugation**

LYNX Rapid Biotin (type 1) Antibody Conjugation Kit was used in this procedure. 1 µl of the Modifier reagent was added to the antibody sample for every 10 µl of antibody and mixed gently. The mixed antibody-modifier sample was transferred directly to the LYNX lyophilized mix and gently resuspended. The mix was incubated at room temperature (20-25°C) for 3 h. After incubation, 1 µl of Quencher reagent was
added for every 10 µl of antibody used and incubated for 30 min before use. Conjugated antibody was stored at 4°C.

5.2.4. GH-V ELISA development

A sandwich ELISA was used in the GH-V assay due to its high efficiency and specificity, in which the antigen is “sandwiched” between two primary antibodies (capture and detection) and then detected using a labelled streptavidin. The advantage of sandwich ELISA is that the sample does not have to be purified before analysis, and the assay is more sensitive than a direct or indirect ELISA. The goals for developing the GH-V ELISA were that the assay should have a maximized signal-to-noise ratio and therefore increased sensitivity. It should be a robust, reproducible assay for testing samples, and be able to measure GH-V over a biologically relevant range.

Capture and detection antibodies

A sandwich ELISA measures the amount of antigen between two layers of antibodies: capture and detecting antibody. The antibodies need to have high specificity and high affinity, and should be matched pairs, which means the capture antibody and detecting antibody bind to two different antigenic epitopes and do not interfere with each other.

In humans, GH-N, GH-V and PL are secreted into maternal circulation during pregnancy. These proteins are encoded from the same gene cluster and share over 90% similarity in amino acid sequence. In order to measure maternal GH-V levels accurately and reduce false positive results, the specific binding of capture and detection antibodies with GH-V was examined by native western blot (Figure 5.1) and indirect ELISA (Figure 5.2). Both experiments demonstrated that human GH-V antibody E8 specifically bound with GH-V, had minimal cross reactivity with GH-N and no cross reactivity with PL. 7C12 detected GH-V but had some cross reactivity with GH-N and
PL. Therefore, E8 was used as the capture antibody due to its binding specificity for GH-V (Evain-Brion et al., 1994; Igout et al., 1993). 7C12 was used as the detection antibody, and biotinylated.

![Specific binding of human GH-V antibody E8 and 7C12 in native western blot. 50 ng of GH-N, GH-V and PL proteins were loaded. Immunoblotting was performed using GH-V antibody E8 and 7C12, respectively.](image)

![Specific binding of human GH-V antibody E8 and 7C12 using an indirect ELISA.](image)

**Optimizing capture and detection antibody concentrations**

Optimal capture and detection antibody concentrations were determined through standard checkerboard titration procedure (Crowther, 1995). A checkerboard titration experiment was conducted empirically using starting concentrations of 8 µg/ml and 16 µg/ml for capture and detection antibodies respectively, with the antigen (GH-V) at a constant concentration of 5 ng/ml (Figure 5.3). The optimal capture and detection antibody concentrations were 2 µg/ml and 8 µg/ml, respectively. Horseradish peroxidase conjugated streptavidin was used at a concentration of 200 ng/ml as no significant differences were seen at higher or lower concentrations.
Optimizing the blocking buffer

The first step of sandwich ELISA is the immobilization of biomolecules, primarily proteins, to a solid surface. The ability of the surface to interact with proteins and other biomolecules is an essential feature; however, non-specific binding of other proteins or biomolecules during subsequent steps of the assay can be detrimental to the specificity and sensitivity of the assay. Non-specific binding to the surface can be minimized by saturating any unoccupied binding sites with a blocking reagent such as bovine serum albumin (BSA) or non-fat dry milk which are used to reduce non-specific binding. A series of blocking buffers were tested, including 2% BSA, 5% BSA, 2% non-fat dry milk, 5% non-fat dry milk and a commercial blocking buffer (Ultrablock) using a standardised ELISA procedure. Ultrablock was found to have lowest signal-to-noise ratio and was used in subsequent experiments as blocking buffer and sample diluent.

Optimizing signal detection

Colour development time was optimized by comparing 10, 20 and 30 min incubations. An incubation for 20 min provided appropriate colour development with a dynamic range.
**Standard curve**

Recombinant human GH-V was reconstituted in 0.4% NaHCO₃ pH 9 at a concentration of 500 µg/ml, aliquoted and stored at -80°C as the standard stock. A 5 ng/ml standard was prepared from the stock, and a serial dilution was performed to obtain concentrations of 2.5, 1.25, 0.625, 0.313, 0.156 and 0.078 µg/ml. Sample diluent served as blank.

**Spike-and-recovery and linearity-of-dilution**

The sample reading obtained from an ELISA is dependent upon the interaction between the antigen of interest and the antibodies by comparison of this interaction relative to a recombinant protein standard curve. As a heterogenous assay, ELISA separates some components of the analytical reaction mixture by adsorbing certain components onto a solid phase which is physically immobilized. Buffer components, sample matrix, complement, heterophilic antibodies and other factors can interfere with antibody binding in a natural sample and therefore influence the accuracy of ELISA results. Therefore, spike-and-recovery and linearity-of-dilution tests are commonly performed to access the accuracy of ELISA for particular sample types and determine whether analyte detection is affected by different diluents or the biological sample matrix.

In a spike-and-recovery test, a known amount of recombinant protein is spiked into a sample and a recovery rate is calculated. Three sets of unspiked, spiked and control samples were prepared. 0.625 ng/ml GH-V was spiked in a serum sample from a healthy donor and sample diluent to generate a spiked sample and a control sample, respectively. Then serial dilutions (1:2, 1:4 and 1:8) were obtained from unspiked, spiked and control samples. These dilutions were read off the standard curve to determine whether dilutions samples were parallel to the standard curve and whether the values of the sample dilutions were accurate.
The plate was set up as follows (samples were measured in duplicate):

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Blank</td>
<td>Spiked sample (neat)</td>
<td>Control spike (neat)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Standard 1</td>
<td>1:2 Spiked sample</td>
<td>1:2 Control spike</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Standard 2</td>
<td>1:4 Spiked sample</td>
<td>1:4 Control spike</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Standard 3</td>
<td>1:8 Spiked sample</td>
<td>1:8 Control spike</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>Standard 4</td>
<td>Unspiked sample (neat)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>Standard 5</td>
<td>1:2 Unspiked sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>Standard 6</td>
<td>1:4 Unspiked sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>Standard 7</td>
<td>1:8 Unspiked sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Recovery rate was calculated as the following formula:

\[
\text{% Recovery} = \frac{\text{Observed} – \text{Unspiked}}{\text{Expected}} \times 100
\]

(Observed: Spiked sample value; Unspiked: Unspiked sample value; Expected: Amount spiked into sample)

The average recovery rate was 106%. Linearity was determined by calculating the recovery rate of 1:2, 1:4 and 1:8 dilutions spiked and unspiked samples and fell within the acceptance range of 80-120% (Table 5.1).

Table 5.1: Recovery rate of 1:2, 1:4 and 1:8 dilutions spiked and unspiked samples.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Spiked samples</th>
<th>Unspiked samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:2</td>
<td>98.3%</td>
<td>90.7%</td>
</tr>
<tr>
<td>1:4</td>
<td>110.2%</td>
<td>101.2%</td>
</tr>
<tr>
<td>1:8</td>
<td>93.5%</td>
<td>111.9%</td>
</tr>
</tbody>
</table>

Parallelism was used to determine whether natural and recombinant samples are detected in the given ELISA in similar fashion. The result from the parallelism validation ensures that the analyte of interest is detected in a natural sample in a dose-dependent manner similar to the standard curve without matrix interfering effects. Standards and serum samples were serially diluted. All diluted samples were measure
three times. The optical density (OD) of each dilution was plotted against the dilution factor. Parallelism between the GH-V standards and serial dilutions of serum samples indicated that the standard accurately reflected the GH-V content in natural samples (Figure 5.4).

![Graph of delta OD vs. dilution factor](image)

**Figure 5.4:** Comparison of the optical density of standard and serum sample dilutions.

**Intra- and Inter-assay coefficient of variation**

The coefficient of variation (CV) is used to express the precision and repeatability of an assay. The CV for a sample is defined as the ratio of the standard deviation to the mean. The intra-assay variation describes the variation of results within a data set obtained from one experiment. Each sample was measured three times in a plate, and then CV was calculated for each sample. The average of the individual CVs was denoted as intra-assay CV. The inter-assay variation describes the variation of results obtained from repeated experiments and is an expression of plate-to-plate consistency. Similarly, each sample was measured in different plates, then CV was calculated, and the average of the individual CVs was denoted as inter-assay CV. The intra- and inter-assay CV for the GH-V ELISA was 4.8% and 6.8%, respectively. Exemplary determinations are represented in Table 5.2 and 5.3.
Table 5.2: Intra-assay CV

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean value (ng/ml)</td>
<td>2.38</td>
<td>1.50</td>
<td>4.18</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.04</td>
<td>0.04</td>
<td>0.11</td>
</tr>
<tr>
<td>CV (%)</td>
<td>1.72</td>
<td>2.39</td>
<td>2.72</td>
</tr>
</tbody>
</table>

Table 5.3: Inter-assay CV

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean value (ng/ml)</td>
<td>5.59</td>
<td>2.52</td>
<td>3.89</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.33</td>
<td>0.16</td>
<td>0.31</td>
</tr>
<tr>
<td>CV (%)</td>
<td>5.93</td>
<td>6.29</td>
<td>7.73</td>
</tr>
</tbody>
</table>

5.3. Maternal serum GH-V in inappropriate birth weight for gestational age pregnancies

5.3.1. Introduction

Delivery of infants with an appropriate birth weight for gestational age is a goal of obstetric care. However, inappropriate fetal growth, either LGA or SGA, is common and clinically relevant as they are linked with a number of perinatal complications. LGA is associated with higher rates of Caesarean birth, shoulder dystocia, postpartum haemorrhage and neonatal hypoglycaemia (Weissmann-Brenner et al., 2012), while SGA infants are more likely to be stillborn, and develop perinatal asphyxia, hypothermia and abnormal neurologic symptoms (Doctor et al., 2001; Flenady et al., 2011). Furthermore, both LGA and SGA have been reported to increase the risk for developing certain diseases in later life, such as obesity, type 2 diabetes, hypertension, and dyslipidemia (Barker et al., 1990; Eriksson et al., 2001; Gluckman and Hanson, 2004; Lithell et al., 1996).
Previous studies have demonstrated a positive relationship between maternal GH-V serum concentration and fetal growth (Chellakooty *et al.*, 2004; Handwerger and Freemark, 2000; Koutsaki *et al.*, 2011; McIntyre *et al.*, 2000; Mirlesse *et al.*, 1993; Mittal *et al.*, 2007a; Pedersen *et al.*, 2010). Consistently, decreased concentrations of circulating GH-V, and reduced placental mRNA expression of this hormone, have been observed in mid to late stage growth restricted pregnancies, compared to normal pregnancies (Koutsaki *et al.*, 2011; McIntyre *et al.*, 2000). However, another study showed that there was no significant difference in maternal GH-V at 11-13 weeks in SGA pregnancies compared to non-SGA ones (Sifakis *et al.*, 2012c). Therefore, whether there is an association between maternal concentration of GH-V earlier in pregnancy and infant birth weight at term is not clear. In the present study, I aimed to examine the relationship between maternal serum GH-V concentrations at 20 weeks of gestation and infant birth weight. I hypothesised that maternal serum GH-V concentrations were altered in pregnancies with inappropriate birth weight and maternal GH-V was positively related to birth weight. Maternal serum IGF-1, IGF-2, IGFBP-1 and IGFBP-3 concentrations were also measured to investigate potential relationships between GH-V and other primary components of the IGF-IGFBP system.

5.3.2. Materials and Methods

Ethical approval was obtained from New Zealand Health and Disability Ethics Committees (AKX/02/00/364/AM03), and all women provided written informed consent. Between November 2004 and October 2007, 2,032 nulliparous women with singleton pregnancies were recruited to the Screening for Pregnancy Endpoints (SCOPE) study in Auckland, New Zealand. The inclusion criteria has been described previously (McCowan *et al.*, 2007).
Participants were interviewed and examined by a SCOPE research midwife at 15 and 20 weeks of gestation. At the first visit, detailed clinical and demographic data were collected and entered into an internet accessed, central database with a complete audit trail (MedSciNet, Stockholm, Sweden). Umbilical artery resistance index (RI, defined as maximum – minimum velocity/maximum velocity) and mean uterine artery RI were measured using Doppler ultrasound at 20 weeks. Maternal serum samples were collected at 20 weeks and stored at -80°C for subsequent analyses. Birth weight was recorded using electronic scales at the time of birth.

In this nested case-control study, 50 LGA and 49 SGA cases were selected and matched to 50 controls (appropriate-for-gestational age; AGA), matched by ethnicity. No women included in this study had pregnancies complicated by gestational diabetes or gestational hypertensive disease. Customised birth weight centile was calculated, adjusted for mother’s height and weight at 15 weeks’ visit, ethnicity, sex and gestation at delivery. SGA and LGA were defined as birth weight <5th and >95th customized birth weight centiles, respectively (McCowan et al., 2004).

The GH-V ELISA procedure

Microtiter plates were coated with antibody E8 diluted in phosphate buffer (0.1 M Sodium Carbonate, pH 9.5) at a concentration of 2 µg/ml by overnight incubation at 4°C. Coated plates were washed three times with wash buffer (PBS-T; 10 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.05% Tween 20). Blocking was achieved by 1 hour incubation at room temperature with Ultrablock. Standards were prepared from GH-V solution with a range from 0.078 to 5.0 ng/ml. Standards and neat serum samples were incubated for 2 hours at room temperature, then washed three times. All serum samples were measured in duplicate. Biotinylated 7C12 (8 µg/ml) was added and incubated for 1 hour. The plates were washed three times and 200 ng/ml horseradish...
peroxidase conjugated streptavidin was added and incubated for 30 min. The microtiter plates were then washed four times and 3, 3', 5, 5'-Tetramethylbenzidine (TMB) Substrate Reagent Set (BD Biosciences) (van der Lely and Kopchick, 2006) was added to the wells producing a visible signal that is correlated with the amount of antigen. Absorbance was read at 450nm and 590 nm within 30 min of stopping the reaction.

**Serum analysis**

Serum IGF-1, IGF-2, IGFBP-1 and IGFBP-3 were assayed with human-ELISA as per the manufacturer’s instructions (Mediagnost, Germany).

**Statistical analysis**

Concentrations of GH-V, IGF-2, IGFBP-1, and IGFBP-3 were positively skewed. Outliers were defined as data points more than 1.5x the interquartile range above the upper or below the lower quartile. Data were log-transformed to improve the approximation of normal distribution and linearize relationships. Data are expressed as means ± S.E.M or median unless stated otherwise. Group means were compared with one way ANOVA with post-hoc analysis (Tukey's procedure). Categorical and numerical variables were compared using chi-square or Fisher’s exact test. Pearson’s coefficient was used to determine correlations between variables, presented as r values. Linear regression analysis was used to explore the relationships between two variables. Multiple regression was used to determine the association of maternal hormone concentrations with birth weight after controlling for known clinical correlates. All analyses were conducted using IBM SPSS Statistics 21. A p-value of <0.05 was accepted as statistically significant.

**5.3.3. Results**

The demographic and clinical details of the three groups are shown in Table 5.4. SGA babies were born earlier with smaller placentas. The mean uterine artery RI at 20 weeks
was significantly higher in women destined to deliver SGA neonates (mean uterine artery RI, 0.61) than in women with AGA (0.56) (p < 0.05) or LGA pregnancies (0.52) (p < 0.05). However, there was no significant difference in umbilical artery RI between groups.

Table 5.4: Demographic and clinical findings in AGA, LGA and SGA cases.

<table>
<thead>
<tr>
<th></th>
<th>AGA n=50</th>
<th>LGA n=50</th>
<th>SGA n=49</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal age (yrs)</td>
<td>29.1 (0.8)</td>
<td>30.5 (0.6)</td>
<td>29.8 (0.7)</td>
<td>ns</td>
</tr>
<tr>
<td>Socioeconomic index*</td>
<td>47 (2)</td>
<td>50 (2)</td>
<td>48 (2)</td>
<td>ns</td>
</tr>
<tr>
<td>Caucasian, n (%)</td>
<td>38 (76.0)</td>
<td>38 (76.0)</td>
<td>38 (77.6)</td>
<td>ns</td>
</tr>
<tr>
<td>Smoked at 15 wks, n (%)</td>
<td>5 (10.0)</td>
<td>1 (2.0)</td>
<td>5 (10.2)</td>
<td>ns</td>
</tr>
<tr>
<td>Any alcohol intake at 15 wks, n (%)</td>
<td>2 (4.0)</td>
<td>1 (2.0)</td>
<td>5 (10.2)</td>
<td>ns</td>
</tr>
<tr>
<td>BMI at 15 wks (kg/m2)</td>
<td>25.1 (0.6)</td>
<td>24.8 (0.6)</td>
<td>23.9 (0.6)</td>
<td>ns</td>
</tr>
<tr>
<td>Umbilical artery RI at 20 wks</td>
<td>0.72 (0.01)</td>
<td>0.72 (0.01)</td>
<td>0.74 (0.01)</td>
<td>ns</td>
</tr>
<tr>
<td>Mean uterine artery RI at 20 wks</td>
<td>0.56 (0.01)$^a$</td>
<td>0.51 (0.01)$^a$</td>
<td>0.61 (0.01)$^b$</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Gestational age at delivery (wks)</td>
<td>40.1 (0.2)$^a$</td>
<td>39.5 (0.2)$^{ab}$</td>
<td>39.2 (0.4)$^b$</td>
<td>0.0373</td>
</tr>
<tr>
<td>Fetal characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3535 (46)$^{a}$</td>
<td>4279 (43)$^{b}$</td>
<td>2518 (75)$^{c}$</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Placental weight (g)</td>
<td>640 (18)$^a$</td>
<td>825 (24)$^b$</td>
<td>483 (18)$^c$</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>35 (70.0)</td>
<td>31 (62.0)</td>
<td>31 (63.3)</td>
<td>ns</td>
</tr>
</tbody>
</table>

Results expressed as mean (SEM) if not indicated; ns, not significant;

P values are for overall comparison of the 3 groups; Groups which do not share the same letter are significantly different from each other (p <0.05).

* Socioeconomic index calculated using the New Zealand Socioeconomic Index guide (1996).

Concentrations of maternal hormones

Maternal serum GH-V concentrations varied between individuals. A few samples had absorbance values falling out of the range of the standard curve. The concentration of these samples was extrapolated from the standard curve, as insufficient samples were available for repeat measurements. Subsequently those extrapolated data were
identified as outliers and were not included in the group comparisons (Figure 5.5A and B). Maternal serum GH-V concentrations at 20 weeks of gestation were significantly higher in LGA pregnancies (median concentration, 2.07 ng/ml) compared to AGA (1.72 ng/ml) and SGA pregnancies (1.65 ng/ml) (p < 0.05), however, there was no significant difference between SGA and AGA samples (Figure 5.5A and B). There were no significant differences in IGF-1, IGF-2, IGFBP1 and IGFBP-3 concentrations between the groups (Figure 5.5C, D, E and F). Infant gender did not affect the maternal concentrations of GH-V, IGFs and IGFBPs at 20 weeks.
Figure 5.5: Serum GH-V, IGF-1, IGF-2, IGFBP-1 and IGFBP-3 concentrations in AGA, LGA and SGA cases. Data are showed as Tukey box-whisker plots (median, 25th centile, 75th centile and range). Groups which do not share the same letter are significantly different from each other (p<0.05). Outliers are presented as hollow symbols.
Correlation analysis

One of the aims of this study was to determine whether estimations of maternal GH-related parameters were related to birth weights (Table 5.5). In the correlation analysis there was a weak but significant positive relationship of maternal GH-V concentrations with birth weights ($r = 0.176, p = 0.033$) (Figure 5.6A), birth weights adjusted for gestational age ($r = 0.174, p = 0.035$) and customised birth weight centiles ($r = 0.163, p = 0.046$) (Table 5.5), as well as placental weights ($r = 0.233, p = 0.011$). The mean uterine artery RI at 20 weeks was negatively associated with birth weight ($r = -0.414, p < 0.0001$), birth weight adjusted for gestational age ($r = -0.402, p < 0.0001$) and customised birth weight centile ($r = -0.38, p < 0.0001$). Maternal IGF-1 concentrations were related to the changes in GH-V ($r = 0.343, p < 0.0001$) (Figure 5.6B), the changes in IGF-2 ($r = -0.168, p = 0.042$) and the changes in IGFBP-3 ($r = 0.187, p = 0.027$). There were no correlations between maternal IGF-I, IGF-2, IGFBP-3 and birth weights. However, maternal IGFBP-1 showed a weak positive relationship with mean uterine artery RI ($r = 0.258, p = 0.002$) and a weak inverse relationship with birth weights ($r = -0.257, p = 0.002$) (Figure 5.6C), birth weights adjusted for gestational age ($r = -0.253, p = 0.002$) and customised birth weight centiles ($r = -0.210, p = 0.011$) (Table 5.5).
Table 5.5: Univariate linear regression models with birth weight or customised birth weight centile used as the dependent variable. Values of GH-V, IGF-2, IGFBP-1 and IGFBP-3 here are log-transformed.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>( r )</th>
<th>( r^2 )</th>
<th>95% CI</th>
<th>t</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GH-V</td>
<td>0.176</td>
<td>0.027</td>
<td>12.61;1063</td>
<td>2.024</td>
<td>0.045</td>
</tr>
<tr>
<td>IGF-1</td>
<td>0.124</td>
<td>0.015</td>
<td>-0.52;3.66</td>
<td>1.489</td>
<td>0.139</td>
</tr>
<tr>
<td>IGF-2</td>
<td>0.018</td>
<td>0</td>
<td>-2167;2692</td>
<td>0.214</td>
<td>0.831</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>-0.257</td>
<td>0.066</td>
<td>-1327;-305.6</td>
<td>-3.16</td>
<td>0.002</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>0.013</td>
<td>0</td>
<td>-603.4;705.7</td>
<td>0.154</td>
<td>0.877</td>
</tr>
<tr>
<td>Customised centile</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GH-V</td>
<td>0.163</td>
<td>0.027</td>
<td>0.46;57.49</td>
<td>2.008</td>
<td>0.046</td>
</tr>
<tr>
<td>IGF-1</td>
<td>0.129</td>
<td>0.017</td>
<td>-0.23;0.20</td>
<td>1.566</td>
<td>0.120</td>
</tr>
<tr>
<td>IGF-2</td>
<td>0.058</td>
<td>0.003</td>
<td>-8.46;177.7</td>
<td>0.703</td>
<td>0.483</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>-0.210</td>
<td>0.044</td>
<td>-64.65;-8.42</td>
<td>-2.569</td>
<td>0.011</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>0.017</td>
<td>0</td>
<td>-31.63;39.08</td>
<td>0.208</td>
<td>0.835</td>
</tr>
</tbody>
</table>

The 95% confidence intervals (CI) for beta-coefficient are given.
Figure 5.6: Associations of GH-related hormones and birth weight. A: The association of maternal GH-V concentration and birth weight. B: The association of GH-V and IGF-1. C: The association of maternal IGFBP-1 concentration and birth weight. GH-V and IGFBP-1 concentrations here are log-transformed.
Multiple regression was used to determine whether any GH related variables were associated with birth weight after controlling for maternal age, ethnicity, socioeconomic status, smoking and drinking habits, maternal BMI, infant sex and gestation at delivery. Maternal GH-V and IGFBP-1 concentrations were significantly associated with birth weights in separate models that controlled for those factors. Using GH-V and IGFBP-1 in combination with each other, or in combination with the IGF-1, IGF-2 or IGFBP-3 did not improve the model.

5.3.4. Discussion

In the present study, maternal GH-V, IGF-1, IGF-2, IGFBP-1 and IGFBP-3 concentrations were investigated in the maternal samples in pregnancies at 20 weeks of gestation that later resulted in AGA, SGA or LGA births. This approach has allowed determination of the correlation between GH-related hormones and their relationships with birth weight. In this study it was found that maternal serum GH-V was increased at 20 weeks in LGA pregnancies, and that GH-V was positively associated with birth weight.

Most of the studies in mid-late pregnancy found a positive association between maternal GH-V and fetal growth. Chellakooty et al. observed that GH-V concentration at 24.5–37.5 weeks was positively associated with fetal growth rate and birth weight (Chellakooty et al., 2004). Two studies reported lower GH-V concentrations at approximately 30 weeks in pregnancies complicated by fetal growth restriction (McIntyre et al., 2000; Mirlesse et al., 1993). Women with PE and SGA have been shown to have lower maternal serum concentrations of GH-V than women with PE but without SGA (Mittal et al., 2007a).
As mentioned above, GH-V becomes the dominant circulating form of GH after 20 weeks of gestation. Limited studies have investigated associations of GH-V with fetal growth at earlier time points. Pedersen et al. observed increased GH-V concentration in women at weeks 11-14 carrying fetuses with high growth rates assessed by sonographic measurements (Pedersen et al., 2010). However, another study found that maternal GH-V at that period was not associated with birth weight in either SGA or normal pregnancies (Koutsaki et al., 2011). Studies that showed no relationship may have been limited by issues of sample size and/or the time of sampling. This study provides further evidence that maternal GH-V concentration at mid-gestation, as early as 20 weeks, has a positive relationship with birth weight. However, both LGA and SGA can either occur following a pathological process or may represent constitutionally big or small infants. The definitions of LGA and SGA cannot distinguish one process from the other, and those infants in the AGA range are presumably healthy. Further, GH-V has been found to be associated with a number of pathological conditions, such as PE (Mittal et al., 2007a), gestational diabetes (McIntyre et al., 2000), and Down's syndrome (Baviera et al., 2004). In this study, an association was found of GH-V with birth weight in LGA, AGA and SGA cases. Whether this association reflects the “normal” or pathological changes of birth weight is still unclear.

GH-V is thought to play a key role in maternal adaptation to pregnancy and fetal growth (Newbern and Freemark, 2011). Firstly, GH-V promotes the adaptation to pregnancy of blood vessels supplying the placenta (Lacroix et al., 2005), and relaxes the arteries supplying the uterus (Schiessl et al., 2007); the effect of these changes is an increase in blood flow to the fetus. Secondly, GH-V shares similar physiological somatotrophic, lactogenic and lipolytic properties with GH-N (Alsat et al., 1997; Verhaeghe, 2008). The growth-promoting effect of GH-V has also been demonstrated
in vivo in non-pregnant hypophysectomized rats treated with GH-V and transgenic mice (Barbour et al., 2002; MacLeod et al., 1991b; Selden et al., 1988a). Thirdly, GH-V increases maternal concentrations of other important growth factors, such as IGF-1 (Caufriez et al., 1990a; Caufriez et al., 1994). A highly significant correlation between GH-V and IGF-1 was also found in the present study. Moreover, GH-V has been proposed to be a likely candidate to mediate the insulin resistance of pregnancy in transgenic mice (Barbour et al., 2002), where GH-V may impact on maternal metabolism and substrate supply to the fetus, either directly or mediated by IGF-1.

IGF-1 and IGF-2 mediate a range of actions in many tissues including stimulation of cell growth, cell survival and differentiation. These actions are regulated by a series of specific binding proteins, which may inhibit or enhance IGF activity (Baxter, 2000). There is clear evidence that the IGFs and IGFBP family are closely related to fetal growth (Boyne et al., 2003; Chard, 1994; Han et al., 1996b; Hills et al., 1996; Holmes et al., 1997), however, their associations were mostly determined at mid to late pregnancy. In the present study, maternal serum IGFBP-1 was negatively related to birth weight as early as 20 weeks, but IGF-1, IGF-2 and IGFBP-3 had no association with birth weight at that time. Maternal serum IGFBP-1 may therefore be a potential biochemical marker for early detection of inappropriate fetal growth.

IGFBP-1 binds to only a small proportion of circulating IGF-1 (Frystyk et al., 2002). However, it is considered to be important for short-term regulation of IGF bioactivity, since IGFBP-1 concentrations fluctuate in response to insulin and carbohydrate intake (Baxter, 1995a). Previous studies have demonstrated two major roles of IGFBP-1. IGFBP-1 serves as an endocrine factor to regulate the bioavailability of serum IGF-1, inhibits IGF binding to cell surface receptors, and thereby inhibits IGF-mediated cell mitogenic and metabolic actions. Such a mechanism can be reconciled with
observations showing that serum concentrations of ‘free’ IGF-1 are markedly increased during late pregnancy, consequent on IGFBP-3 proteolysis and decreased ternary complex formation (Skjaerbaek et al., 2004). The IGF inhibitory actions of IGFBP-1 have been confirmed by in vitro studies and in vivo animal investigations (Jones et al., 1991; Lee et al., 1993; Lowman et al., 1998; Rajkumar et al., 1995). Overexpression and subsequent excess levels of circulating IGFBP-1 result in inhibition of fetal growth and the metabolic effects of the IGFs (Murphy et al., 1995; Rajkumar et al., 1995), while low IGFBP-1 concentrations are associated with macrosomia and insulin resistance syndromes (Heald et al., 2001; Janssen et al., 1998; Yan-Jun et al., 1996). Consistent with previous studies (Harrington et al., 1997b; Melchiorre et al., 2009), we found that uterine artery RI at 20 weeks was increased in pregnancies with SGA and was negatively associated with birth weight. In addition, a positive relationship between IGFBP-1 and uterine artery RI was observed. A recent study revealed that IGFBP-1 induced vasodilatation independently of IGF by increasing endothelial nitric oxide synthase activity in mice (Rajwani et al., 2012). However, the effect of IGFBP-1 on the utero-placental vessel tone during pregnancy is still unclear. The other role of IGFBP-1 is to act as an autocrine/paracrine factor in the female reproductive system (Rutanen and Seppala, 1992). In humans, IGFBP-1 is synthesised in large amounts by the secretory endometrium, ovarian granulosa cells and decidualized stromal endometrial cells of early pregnancy (Koistinen et al., 1990; Rutanen et al., 1985). IGFBP-1, together with IGFs, ovarian steroids and other factors, are involved in a complex system which regulate decidualization, trophoblast invasion, and fetal growth (Hamilton et al., 1998; Han et al., 1996b).

In conclusion, maternal serum GH-V and IGFBP-1 concentrations at 20 weeks of gestation were significantly associated with birth weight. However, given the strength
of the association, a potential clinical use as early markers for aberrant fetal growth patterns would require further investigation. As an association between maternal circulating GH-V and SGA has been observed later in the third trimester, measurements taken later in pregnancy may be more informative.

5.4. Maternal serum GH-V in pregnancies complicated with gestational diabetes mellitus

5.4.1. Introduction

GDM, defined as glucose intolerance with onset or first recognition during pregnancy, represents a failure to maintain normal glucose tolerance during the metabolic stress of pregnancy (Colagiuri et al., 2014). It affects up to 14% of all pregnancies worldwide (King, 1998), and is associated with multiple gestational complications, includingmacrosomia, dystocia, stillbirth, hypoglycaemia and respiratory distress (Langer et al., 2005). Both the fetus and the mother have increased risk for metabolic disorders and diabetes in later life (Catalano et al., 2003).

Previous studies and studies in Chapter 3 and 4 have demonstrated that transgenic expression or administration of exogenous GH-V causes insulin resistance in mice (Barbour et al., 2002; Liao et al., 2016a; Liao et al., 2016b). Further, it is thought that GH-V increases maternal concentrations of other important growth factors, such as IGF-1 as serum concentrations during pregnancy are highly correlated (Caufriez et al., 1990a; Caufriez et al., 1993, 1994). IGFs and IGFBPs affect maternal metabolism, and act as endocrine signals to enhance placental function and fetal growth (Chard, 1994; Forbes and Westwood, 2008). The structural homology between IGFs and insulin and the hypoglycemic activity regulated by IGFBPs suggest that IGFs and their binding
proteins have an intrinsic role in glucose metabolism and homeostasis (Holt et al., 2003). Limited studies implicate GH-V as a potential biomarker of GDM (Fuglsang et al., 2003; McIntyre et al., 2000). Dysregulations of IGFs and their binding proteins were also observed in diabetic pregnancies (Matuszek et al., 2011; Yan-Jun et al., 1996; Zhu et al., 2016).

In this study, I aimed to determine whether there was an association with maternal serum concentration of GH-V at 20 weeks of gestation and the subsequent pregnancy complication of GDM. I hypothesised that maternal serum GH-V concentrations would be altered in pregnancies with GDM. Serum concentrations of related GH/IGF-1 axis proteins: IGF-1, IGF-2, and their binding proteins IGFBP-1 and IGFBP-3 were also measured.

5.4.2. Materials and Methods

In SCOPE study, participants were interviewed and examined by a SCOPE research midwife at 15 and 20 weeks of gestation. At the first visit, detailed clinical and demographic data were collected and entered into an internet accessed, central database with a complete audit trail (MedSciNet, Stockholm, Sweden). Maternal serum samples were collected at 20 weeks and stored at -80°C for subsequent analyses. The specimens did not undergo any freeze/thaw cycles prior to these analyses. Birth weight was recorded using electronic scales at the time of birth. Customised birth weight centile, adjusted for mother’s height and weight at 15 weeks’ visit, ethnicity, sex and gestation at delivery, was calculated to allowed comparison of relative fetal growth across a range of gestational ages. SGA and LGA in this study were defined as birth weight < 10th and > 90th customized birth weight centiles, respectively. All participants underwent the glucose screening (50 g polycose) from 15 to 28 weeks depending on
risk factors. Participants who had abnormal glucose screening results or who were at high risk of developing GDM were advised to perform a two-hour, 75 g oral glucose tolerance test (OGTT). GDM for the purposes of this case control study was diagnosed by International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria based on Hyperglycemia and Adverse Pregnancy Outcome (HAPO) data (fasting plasma glucose $\geq$ 5.1 mmol/L or 1h glucose $\geq$ 10.0 mmol/L or 2h glucose $\geq$ 8.5 mmol/L) (Simmons et al., 2008).

In this nested case-control study, 28 GDM cases were selected from the New Zealand SCOPE cohort and matched by ethnicity, age (± 3 years) and BMI (± 3 kg/m2) to 28 controls.

**Serum analysis**

Maternal serum concentrations of GH-V, IGF-1, IGF-2, IGFBP-1 and IGFBP-3 were measured as described before.

**Statistical analysis**

GH-V data was log-transformed to improve the approximation of normal distribution and linearize relationships. Data are expressed as means ± S.E.M and median unless stated otherwise. Repeated measures were compared using a Student’s t test. Categorical and numerical variables were compared using chi-square or Fisher’s exact test. Pearson’s coefficient was used to determine correlations between variables, presented as r values. Multivariate linear regression analysis was adopted to determine the association of maternal hormone concentrations with changes in glucose levels. All analyses were conducted using IBM SPSS Statistics 21. A p-value of <0.05 was accepted as statistically significant.

**5.4.3. Results**

The demographic and clinical details are shown in Table 5.6.
Table 5.6: Demographic and clinical findings in GDM and control cases.

<table>
<thead>
<tr>
<th></th>
<th>GDM n=28</th>
<th>Control n=28</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maternal characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal age (yrs)</td>
<td>31.4 (0.9)</td>
<td>31.3 (0.8)</td>
<td>ns</td>
</tr>
<tr>
<td>Socioeconomic index*</td>
<td>47 (3)</td>
<td>48 (3)</td>
<td>ns</td>
</tr>
<tr>
<td>Caucasian, n (%)</td>
<td>18 (64.3)</td>
<td>18 (64.3)</td>
<td>ns</td>
</tr>
<tr>
<td>Family history of diabetes</td>
<td>10 (35.7)</td>
<td>6 (21.4)</td>
<td>ns</td>
</tr>
<tr>
<td>Smoked at 15 wks, n (%)</td>
<td>3 (10.7)</td>
<td>5 (17.9)</td>
<td>ns</td>
</tr>
<tr>
<td>Any alcohol intake at 15 wks, n (%)</td>
<td>2 (7.1)</td>
<td>2 (7.1)</td>
<td>ns</td>
</tr>
<tr>
<td>BMI at 15 wks (kg/m²)</td>
<td>27.2 (0.9)</td>
<td>26.2 (0.8)</td>
<td>ns</td>
</tr>
<tr>
<td>Gestational age at delivery (wks)</td>
<td>38.2 (0.4)</td>
<td>39.6 (0.2)</td>
<td>0.0054</td>
</tr>
<tr>
<td><strong>Infant characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3449 (101)</td>
<td>3460 (85)</td>
<td>ns</td>
</tr>
<tr>
<td>Customised birth weight centile (%)</td>
<td>60.2 (5.5)</td>
<td>51.8 (5.3)</td>
<td>ns</td>
</tr>
<tr>
<td>SGA at birth</td>
<td>2 (7.1)</td>
<td>0 (0)</td>
<td>ns</td>
</tr>
<tr>
<td>LGA at birth</td>
<td>7 (25)</td>
<td>4 (14.3)</td>
<td>ns</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>16 (57.1)</td>
<td>15 (53.6)</td>
<td>ns</td>
</tr>
</tbody>
</table>

Results expressed as mean (SEM) if not indicated; ns, not significant.

* Socioeconomic index calculated using the New Zealand Socioeconomic Index guide (1996).

**Concentrations of maternal GH-related hormones**

There was no significant difference in maternal serum GH-V concentration in the GDM group at 20 weeks of gestation, when compared to the control group (median concentration, 1.57 ng/ml vs. 1.30 ng/ml, p = 0.079) (Figure 5.7A). However, GDM patients who delivered LGA babies had significantly higher serum GH-V concentrations compared to non-diabetic control cases (median concentration, 2.09 ng/ml vs. 1.30 ng/ml, p = 0.02). Maternal serum IGF-1 concentration in GDM pregnancies was significantly higher than in the control group (median concentration, 273.4 ng/ml vs. 201.1 ng/ml, p < 0.0001) (Figure 5.7B). Maternal serum IGFBP-1 concentration was significantly lower in GDM pregnancies than in controls (median
concentration, 40.78 ng/ml vs. 60.42 ng/ml, p = 0.0004) (Figure 5.7D). There was no significant difference in serum IGF-2 or IGFBP-3 concentrations between groups (Figure 5.7C and E).

![Figure 5.7: Serum GH-V, IGF-1, IGF-2, IGFBP-1 and IGFBP-3 concentrations in GDM and control cases. Data are showed as Tukey box-whisker plots (median, 25th centile, 75th centile and range). Outliers are presented as hollow symbols. *p < 0.05.](image-url)
Correlation analysis

In the GDM group, maternal IGF-1 concentration was positively related to the concentrations of GH-V and IGFBP-3 but negatively related to the IGFBP-1 concentrations (Table 5.7). Maternal fasting glucose concentration in OGTT was positively related to IGF-1 ($r = 0.38$, $p = 0.046$) and negatively related to IGFBP-1 ($r = -0.465$, $p = 0.013$), but these correlations were not seen with 1h or 2h glucose concentrations in OGTT measurements. In a multivariate analysis, including maternal age, ethnicity, socioeconomic status, family history of diabetes, smoking and drinking habits, and maternal BMI as explanatory variables, this model eliminated the significant associations of IGF-1 and IGFBP-1 with fasting glucose, as fasting glucose was strongly associated with BMI ($p < 0.001$). There was no correlation between maternal GH-V, IGF-1, IGFBP-1 or IGFBP-3 with customised birth weight centiles in the GDM group.

In the control group, maternal IGF-1 concentration was positively associated with GH-V and IGFBP-3 (Table 5.7). In addition, maternal IGF-1 had a negative relationship with customised birth weight centile ($r = -0.395$, $p = 0.038$).
Table 5.7: Correlations between GH-related hormones in GDM and control groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>r</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GDM group</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-1 vs. GH-V</td>
<td>0.417</td>
<td>0.03</td>
</tr>
<tr>
<td>IGF-2</td>
<td>-0.155</td>
<td>0.432</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>-0.564</td>
<td>0.002</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>0.418</td>
<td>0.027</td>
</tr>
<tr>
<td><strong>Control group</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-1 vs. GH-V</td>
<td>0.511</td>
<td>0.006</td>
</tr>
<tr>
<td>IGF-2</td>
<td>-0.238</td>
<td>0.222</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>-0.348</td>
<td>0.069</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>0.499</td>
<td>0.007</td>
</tr>
</tbody>
</table>

5.4.4. Discussion

Human pregnancy is characterized by a series of metabolic changes that induce a physiologic form of insulin resistance. GDM develops when insulin secretion is inadequate to compensate for this insulin resistance. Thus, chronic insulin resistance is a central component of the pathophysiology of GDM. The rationale for this study is that GH-V was thought to be the primary regulator of IGF-I in normal and abnormal human pregnancies (Caufriez et al., 1993) and be a potential candidate to mediate the insulin resistance of pregnancy (Barbour et al., 2002). As described in Chapter 3, recombinant GH-V treatment of pregnant or non-pregnant female mice reduces maternal insulin sensitivity in dose-dependent manner (Liao et al., 2016a; Liao et al., 2016b). It was therefore hypothesised that the maternal serum GH-V concentration would also be altered in GDM pregnancies. However, a direct diabetogenic effect of GH-V at 20 weeks of gestation was not supported by the present findings.
Limited studies suggest a possible regulatory effect of glucose levels on GH-V secretion. Patel et al. observed a dose-dependent inhibition of GH-V secretion by glucose in human placental explants and in trophoblast cultures (Patel et al., 1995). Bjorklund et al. described an increase in GH-V during a hyperinsulinemic hypoglycemic clamp in pregnant Type 1 diabetes patients (Bjorklund et al., 1998). No studies to date have demonstrated higher levels of GH in diabetic pregnancies. McIntyre et al. found that maternal GH-V concentrations were positively correlated with maternal glycaemia in women with established Type 1 and Type 2 diabetes, particularly in the post prandial state (McIntyre et al., 2000). However, the study by McIntyre et al. and two other studies conducted by Higgins et al. (Higgins et al., 2012) and Verhaeghe et al. (Verhaeghe et al., 2002) failed to show differences between GH-V concentrations in women with normal glucose tolerance and diabetic patients. Furthermore, Fuglsang et al. demonstrated that the increase in insulin requirements during pregnancy in Type 1 diabetes was not related to GH-V levels (Fuglsang et al., 2003; Fuglsang et al., 2005a). In this study, GH-V concentration at 20 weeks’ gestation was not altered in GDM pregnancies.

Although blood glucose levels are regulated primarily by adjustments in insulin concentrations, accumulating evidence indicates a complementary role of IGF-1 through its insulin-like activity (Clemmons, 2004). A family of six IGFBPs has been characterized to prolong IGF half-life in the circulation and regulate IGF-1 bioactivity (Baxter, 1994). Their relationships are complex. IGFBPs not only regulate IGFs bioavailability but also have biological activity that is independent of IGF (Wheatercroft and Kearney, 2009). Moreover, the hypothesis has been made that cleavage of the IGFBPs into fragments with lower affinity to IGFs allows for increased IGF receptor activation (Collett-Solberg and Cohen, 1996). As much as 99% of IGF-1 in the
circulation is bound to IGFBP-3 with an acid-labile subunit to form a 150 kDa ternary complex. However, the exact role of IGFBP-3 in glucose metabolism, either as a protective effect or enhancing effect on insulin resistance, is still unclear (Kim, 2013; Kim et al., 2007; Mohanraj et al., 2013; Yamada et al., 2010). Although it is less abundant than IGFBP-3, IGFBP-1 has been proposed to play an important role in glucose homeostasis as a dynamic regulator of IGF bioactivity (Katz et al., 2002; Lee et al., 1997; Lewitt et al., 1991; Rajkumar et al., 1996). It has been shown that conditions characterized by insulin resistance are associated with decreased IGFBP-1 levels (Buyalos et al., 1995; Mogul et al., 1996). In GDM patients, increased IGF-1 and decreased IGFBP-1 concentrations were observed in maternal serum at mid-late pregnancy (24 weeks onwards) (Hughes et al., 1995; Luo et al., 2012; Ramirez et al., 2014). Qiu et al. also reported that free IGF-1 and IGFBP-1 at 13 weeks had an inverse association with subsequent GDM risk (Qiu et al., 2005). This study provides further evidence for the change of IGFs and IGFBPs at 20 weeks of gestation in GDM pregnancies.

The findings of this study indicate that the maternal serum concentration of placental growth hormone at 20 weeks’ gestation is unlikely to be useful in the early prediction of GDM.

5.5. Maternal serum GH-V in pregnancies complicated with preeclampsia

5.5.1. Introduction

PE is one of the leading causes of maternal, fetal, and neonatal mortality and morbidity. It affects 3–5% of pregnancies worldwide and is characterized by maternal
hypertension, proteinuria, increased vascular injury and permeability, and shallow placental invasion, and if left untreated it can progress to maternal multiorgan failure, coagulopathy and seizures (Friedman et al., 1991a). Women whose pregnancies were complicated by PE also had an increased risk of chronic hypertension, diabetes mellitus, ischemic heart disease, cerebrovascular disease and kidney disease later in life (Amaral et al., 2015; Funai et al., 2005). However, to date, no therapeutic approaches are available for treatment and there are limited options for prevention of PE in women identified to be at high risk (Henderson et al., 2014). Therefore, identification of biomarkers that can predict the development or assist in the detection of this life-threatening pregnancy disorder is warranted.

During pregnancy, GH-V is secreted from the syncytiotrophoblast cells of the placenta, and gradually replaces GH-N as the main form of GH in the maternal circulation (Haig, 2008; Lacroix et al., 2002b). Despite this intriguing switch in expression from pituitary to placental expression of GH, very little is known about the role GH-V plays in human pregnancy. However, it is thought that GH-V plays a role in maternal adaptation to pregnancy. Studies investigating the association between maternal serum GH-V with PE are limited, and results have been conflicting. Two studies observed increased GH-V levels in maternal serum at mid to late gestation in pregnancies complicated by PE (Mittal et al., 2007b; Papadopoulou et al., 2006). Sifakis et al. found no changes in GH-V concentration in maternal serum taken at 11-13 weeks in PE cases when compared to controls (Sifakis et al., 2011b). Mannik et al. demonstrated reduced GH-V expression in placentas from pregnancies complicated by PE (Mannik et al., 2012).

In this study, I aimed to determine whether there was an association with maternal serum concentration of the GH-V at 20 weeks of gestation and the subsequent
pregnancy complication of PE. Serum concentrations of related GH/IGF axis proteins: IGF-1, IGF-2, and their binding proteins IGFBP 1 and 3 were also measured.

5.5.2. Materials and Methods

In SCOPE study, participants were interviewed and examined by a research midwife at 15 and 20 weeks of gestation. At the first visit, detailed clinical and demographic data were collected and entered into an internet accessed, central database with a complete audit trail (MedSciNet, Stockholm, Sweden). A family history of gestational hypertensive disorders was defined as a mother and/or sister(s) who had a history of either gestational hypertension or PE. Mean arterial pressure (MAP) was measured by mercury or aneroid sphygmomanometer at 15 and 20 weeks SCOPE research visits. Umbilical artery RI and mean uterine artery RI were measured using Doppler ultrasound at 20 weeks. Maternal serum samples were collected at 20 weeks and stored at -80°C for subsequent analyses. Birth weight was recorded using electronic scales at the time of birth.

In this nested case-control study, 71 PE cases were selected the New Zealand SCOPE cohort and matched by ethnicity, age (± 3 years) and BMI (± 3 kg/m²) to 71 controls. PE defined as gestational hypertension (systolic blood pressure >= 140 mmHg or diastolic blood pressure >= 90 mmHg on at least 2 occasions 4 h apart after the 20 week visit in previous normotensive women) with either proteinuria (>2+ dipstick or urine protein creatinine ratio >= 30 mg/mmol or 24h urinary protein excretion >= 0.3g/24h) or multisystem disease (thrombocytopenia, renal insufficiency, impaired liver function, pulmonary, cerebral or visual symptoms) (Brown et al., 2001). The PE group was separated into early-onset PE patients (defined as PE that developed before 34
weeks of gestation) and late-onset PE (defined as PE which developed at or after 34 weeks of gestation).

Serum analysis
Maternal serum concentrations of GH-V, IGF-1, IGF-2, IGFBP-1 and IGFBP-3 were measured as described before.

Statistical analysis
GH-V, IGF-1, IGFBP-1 and IGFBP-3 data were log-transformed to improve the approximation of normal distribution and linearize relationships. Data are expressed as means ± S.E.M and median unless stated otherwise in the text. Repeated measures were compared using a Student’s t test. Categorical and numerical variables were compared using chi-square or Fisher’s exact test. Pearson’s coefficient was used to determine correlations between variables, presented as r values. All analyses were conducted using IBM SPSS Statistics 21. A p-value of <0.05 was accepted as statistically significant.

5.5.3. Results
The demographic and clinical details are shown in Table 5.8. There were no significant differences between the two groups except for MAP at 15 and 20 weeks, birth weight and gestational age at birth.
Table 5.8: Demographic and clinical findings in PE and control cases.

<table>
<thead>
<tr>
<th></th>
<th>PE n=71</th>
<th>Control n=71</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maternal characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal age (yrs)</td>
<td>30.3 (0.5)</td>
<td>30.5 (0.5)</td>
<td>ns</td>
</tr>
<tr>
<td>Socioeconomic index*</td>
<td>48 (2)</td>
<td>51 (2)</td>
<td>ns</td>
</tr>
<tr>
<td>Caucasian, n (%)</td>
<td>58 (81.7)</td>
<td>58 (81.7)</td>
<td>ns</td>
</tr>
<tr>
<td>Family history of gestational hypertensive disorders</td>
<td>17 (23.9)</td>
<td>8 (11.3)</td>
<td>ns</td>
</tr>
<tr>
<td>Smoked at 15 wks, n (%)</td>
<td>2 (2.8)</td>
<td>1 (1.4)</td>
<td>ns</td>
</tr>
<tr>
<td>Any alcohol intake at 15 wks, n (%)</td>
<td>3 (4.2)</td>
<td>8 (11.3)</td>
<td>ns</td>
</tr>
<tr>
<td>BMI at 15 wks (kg/m²)</td>
<td>26.6 (0.5)</td>
<td>26.2 (0.5)</td>
<td>ns</td>
</tr>
<tr>
<td>MAP at 15 wks (mmHg)</td>
<td>84.2 (0.9)</td>
<td>79.5 (1.5)</td>
<td>0.0017</td>
</tr>
<tr>
<td>MAP at 20 wks (mmHg)</td>
<td>83.5 (0.9)</td>
<td>80.5 (0.9)</td>
<td>0.0183</td>
</tr>
<tr>
<td>Umbilical artery RI at 20 wks</td>
<td>0.71 (0.01)</td>
<td>0.72 (0.01)</td>
<td>ns</td>
</tr>
<tr>
<td>Mean uterine artery RI at 20 wks</td>
<td>0.56 (0.01)</td>
<td>0.55 (0.01)</td>
<td>ns</td>
</tr>
<tr>
<td>Gestational age at delivery (wks)</td>
<td>37.9 (0.3)</td>
<td>40.2 (0.1)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>Infant characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3026 (87)</td>
<td>3572 (41)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Customised birth weight centile (%)</td>
<td>43.3 (3.8)</td>
<td>51.3 (2.9)</td>
<td>ns</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>37 (52.1)</td>
<td>41 (57.7)</td>
<td>ns</td>
</tr>
</tbody>
</table>

Results expressed as mean (SEM) if not indicated; ns, not significant;
* Socioeconomic index calculated using the New Zealand Socioeconomic Index guide (1996).

**Concentrations of maternal GH-related hormones**

Maternal serum GH-V concentrations varied between individuals (range: 0.69- 8.37 ng/ml). There was no significant difference in maternal serum GH-V concentrations at 20 weeks of gestation in the PE group when compared to the control group (median, 1.78 ng/ml vs. 1.65 ng/ml, p = 0.884) (Figure 5.8A). Maternal IGF-1 and IGFBP-3 concentrations and the IGF-1/IGFBP-3 ratio in PE pregnancies were significantly higher than in control (median, 253.1 ng/ml vs. 204.3 ng/ml, p < 0.0001; 8535 ng/ml vs. 7711 ng/ml, p = 0.0023; 0.032 vs. 0.026, p < 0.0001, respectively) (Figure 5.8B, E.
Maternal IGFBP-1 concentration was significantly lower in PE pregnancies than in control (median, 34.85 ng/ml vs. 48.92 ng/ml, p = 0.0006) (Figure 5.8D). There was no significant difference in IGF-2 concentration between groups (Figure 5.8C).

Early-onset PE patients had increased IGF-1 and IGFBP-1 concentrations compared to late-onset PE patients (IGF-1: median, 313.5 ng/ml vs. 251.3 ng/ml, p =0.0268; IGFBP-1: median 9372 ng/ml vs. 8461 ng/ml, p = 0.0469), although the number of patients in the early-onset PE group was much lower (n= 8 vs. n= 63).
Figure 5.8: Serum GH-V, IGF-1, IGF-2, IGFBP-1, IGFBP-3 concentrations and IGF-1/IGFBP-3 ratio in PE and control cases. Data are showed as Tukey box-whisker plots (median, 25th centile, 75th centile and range). Outliers are presented as hollow symbols. *p < 0.05.

Correlation analysis
In both the control and PE groups, maternal IGF-1 concentrations were positively related to the changes in IGFBP-3 but negatively related to IGFBP-1 concentrations.
There was also an association between the concentrations of GH-V and IGF-1 in the control group, but not in the PE group (Table 5.9).

Table 5.9: Correlations between GH-related hormones in PE and control groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PE group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1 vs. GH-V</td>
<td>-0.131 0.296</td>
<td>0.291 0.015</td>
</tr>
<tr>
<td>IGF-2</td>
<td>-0.251 0.839</td>
<td>0.27 0.823</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>-0.292 0.025</td>
<td>-0.373 0.01</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>0.552 0.0001</td>
<td>0.550 0.0001</td>
</tr>
</tbody>
</table>

Interestingly, in the PE group, maternal IGF-1 had a weak positive association with MAP at 15 and 20 weeks \( (r = 0.276, p = 0.021; r = 0.24, p = 0.046) \); while maternal GH-V was negatively associated with mean uterine artery RI \( (r = -0.367, p = 0.002) \) but not with umbilical artery RI \( (r = 0.043, p = 0.72) \) at 20 weeks. The associations of IGF-1 with MAP and of GH-V with uterine artery RI were still significant after adjusting for maternal age, ethnicity, BMI, family history of gestational hypertensive disorders, smoking and drinking habits.

### 5.5.4. Discussion

The precise aetiology of PE remains elusive. A long standing hypothesis is that PE develops as a consequence of an immunologically-initiated impaired trophoblast invasion, shallow implantation and inadequate remodelling of the uterine spiral arteries,
leading to a high-resistance uteroplacental circulation (Kaufmann et al., 2003; Redman and Sargent, 2005). Subsequent oxidative stress and inflammation in the placenta trigger the aberrant expression of pro-inflammatory, anti-angiogenic and angiogenic factors, leading to a systemic endothelial cell dysfunction and an exaggerated inflammatory response (Lam et al., 2005). Trophoblast migration and invasive capacity has been shown to be modulated by a number of factors, including oxygen concentration (Zhou et al., 1998), interleukin and transforming growth factor (Karmakar and Das, 2002), IGF-2 and IGFBP-1 (Hamilton et al., 1998), epidermal and hepatocyte growth factors (Bass et al., 1994; Cartwright et al., 1999). A potential role of GH-V in the regulation of trophoblast invasion is also suggested by the presence of the GHR in the placenta (Frankenne et al., 1992) and the stimulation of trophoblast invasion by GH-V in vitro (Lacroix et al., 2005). Further, GH-V is a target gene of peroxisome proliferator-activated receptor-γ (PPARγ) and has been proposed to be involved in the PPARγ-mediated inhibition of trophoblast invasion in an autocrine manner (Fournier et al., 2008). It is possible that circulating GH-V concentrations may not reflect the effect of GH-V on trophoblast invasion.

Doppler ultrasonography can assess uteroplacental and fetoplacental blood flows and has been suggested to be an effective screening method for PE as increased uterine artery blood flow resistance had been observed prior to the onset of PE (Guzin et al., 2005; Napolitano et al., 2010; Plasencia et al., 2008). One study by Schiessl et al. demonstrated a strong correlation between decreasing uterine and peripheral arterial resistance and increasing maternal serum GH-V concentration (Schiessl et al., 2007). In the present study, maternal GH-V concentration was negatively associated with mean uterine artery RI in preeclamptic pregnancies, suggesting a potential role of GH-V in the alteration of maternal arterial resistance.
IGF-1 and IGF-2 mediate a range of actions in many tissues including stimulation of cell growth, cell survival and differentiation. During human pregnancy, maternal IGF-1 is believed to originate mainly from the maternal liver, and GH-V has been suggested to be a main regulator of its synthesis as serum concentrations during pregnancy are highly correlated (Caufriez et al., 1990b; Caufriez et al., 1993, 1994). All IGFBPs except IGFBP-1 are markedly reduced by pregnancy (Gargosky et al., 1990). The majority of circulating IGF-1 is bound to IGFBP-3 and the acid labile subunit to form a 150 kDa ternary complex and is the major storage form of IGF-1 in the circulation (Baxter, 1988). This complex prolongs the half-life of circulating IGF-1 and facilitates its endocrine actions. Due to an endogenous pregnancy-related serum IGFBP-3 proteolytic activity, IGFBP-3 markedly decreases during pregnancy, resulting in an increase of the free form of IGF-1 in circulation (Hasegawa et al., 1995). IGF-1/IGFBP-3 ratio has been found to correlate with the amount of free, biologically active IGF-1 (Juul et al., 1994). IGFBP-1 binds to only a small proportion of circulating IGF-I but is considered to be important for short-term regulation of IGF bioactivity (Baxter, 1995b; Frystyk et al., 2002). The placenta expresses considerable amounts of IGFs and IGFBPs; however, it is unclear if the placenta-derived IGFs and IGFBPs serve local function by paracrine or autocrine regulation, or if they are secreted into the maternal or fetal circulation. Nevertheless, IGFs and IGFBPs are crucial for fetal growth and placental development since they regulate trophoblast migration at the maternal-fetal interface (Hamilton et al., 1998; Lacey et al., 2002). In addition, previous studies also provide evidence in support of a potential role of IGFs and IGFBPs in pregnancies complicated by PE. Increased IGF-2 and decreased IGFBP-1 mRNA expression were observed in the placentae of women with PE (Gratton et al., 2002; Shin et al., 2003). Transgenic mice overexpressing human IGFBP-1 exhibit a PE phenotype (Crossey et
al., 2002). However, maternal serum concentrations of IGFs and IGFBPs in PE pregnancies vary across different studies. Maternal serum IGF-1 and IGFBP-1 were decreased and IGFBP-3 was increased at 11-13 weeks in pregnancies that subsequently developed PE in several studies (Sifakis et al., 2010a, 2011a, 2012a). However, two studies observed deceased IGF-1 and IGFBP-3, as well as increased IGFBP-1 levels in the third trimester in PE patients (Ingec et al., 2004; Kocyigit et al., 2004). Consistent with this study, increased IGF-I and decreased IGFBP-1 from the first to second trimester were associated with higher risk of PE (Hietala et al., 2000; Vatten et al., 2008). Further, a progressive increase in maternal circulating IGFBP-1 concentrations from 16 to 36 weeks was also observed in those pregnancies destined to develop PE (Anim-Nyame et al., 2000). Contributing factors to this observed variation may include the sample size, the time of sampling and the onset and severity of PE where placental integrity is compromised, and consequently the secretion of placental hormones is also compromised.

There is clear evidence that maternal MAP in the second trimester is associated with the later development of PE, although the predictive values for blood pressure alone are low (Caritis et al., 1998; Ekholm et al., 1994; Onwudiwe et al., 2008). The vasoactive effects of IGF-1 indicate that IGF-1 can control blood pressure (Pete et al., 1996; Tivesten et al., 2002). Increased IGF-1 levels have also been reported in patients with hypertension (Diez, 1999; Galderisi et al., 2001). In this study, it is found that maternal IGF-1 concentration was positively associated with MAP in the PE group only. However, the role of circulating IGF-1 in the development of PE still needs further investigation.
This study provides further evidence for the change of the IGFs and IGFBPs at 20 weeks of gestation in PE pregnancies. However, maternal GH-V concentration was not altered and is unlikely to be useful in the early prediction of PE.
Chapter 6: Discussion

The GH-IGF axis is one of the major regulators in terms of fetal growth and maternal adaptation during human pregnancy. GH exerts its action in all cells of the body, promoting synthesis of proteins, accumulation of carbohydrates as glycogen and use of energy resources as lipids. The human genomic locus consists of a cluster of five GH/CS genes. The functions of the GH-N, CS-A and CS-B genes are well known. The GH-V gene, which was originally considered to be transcriptionally silent, has been gradually characterized over the last 20 years. GH-V has been shown to stimulate gluconeogenesis, lipolysis and anabolism, thereby increasing nutrient availability for the fetoplacental unit; and is thought to have a direct influence on placental development and/or on syncytiotrophoblast differentiation and function. Human studies also suggest that GH-V might be associated with pathological pregnancies (detailed in Chapter 1). However, the exact physical and pathological effect of GH-V in pregnancy is still largely unknown. This thesis provides further insight into the effects of GH-V during pregnancy using a mouse model and through investigating maternal serum concentrations in complicated human pregnancies, and investigates potential mechanisms involved.

In Chapter 3, an in vivo study was conducted to determine the dose-response relationship for human GH-V treatment in a mouse model of normal pregnancy. I found that at higher doses, GH-V treatment can cause hyperinsulinemia and is a likely mediator of the insulin resistance associated with late pregnancy. Hepatic expression of the gene for Glut4 was significantly down-regulated after GH-V treatment, which may contribute to the insulin resistance induced by GH-V. Unlike pituitary GH, the secretion of GH-V from placenta is non-pulsatile. This continuous secretion by villous
Syncytiotrophoblast into the maternal compartment might alter maternal metabolism and affect fetal growth during pregnancy.

In Chapter 4, I reported for the first time that the effect of GH-V in different administration methods. The results showed significant differences between different maternal GH-V concentration profiles caused by delivery modes. Both pulsatile and continuous GH-V treatment caused insulin resistance and increased hepatic GHR mRNA expression. While pulsatile GH-V treatment was more effective in stimulating growth but caused marked hyperinsulinemia in mice. These findings suggest GH-V effects on growth and carbohydrate metabolism, implicating that GH-V kinetics in maternal blood may play a role in fetal growth and the development of gestational diabetes.

In Chapter 5 maternal serum concentrations of GH-V, IGFs and IGFBPs at 20 weeks of gestation were investigated in inappropriate fetal growth, GDM and PE pregnancies in nested case-control studies. An increase in the maternal serum GH-V concentration was observed in LGA pregnancies compared to AGA or SGA pregnancies. In addition, maternal serum GH-V and IGFBP-1 at 20 weeks' gestation was associated with fetal growth. These results support a role of this axis in fetal growth. However, maternal serum GH-V concentrations were not altered in GDM and PE pregnancies.

Strengths and limitations of the individual studies that comprise this body of work have been presented in previous chapters reporting each study. The prime limitation of the current investigation is the use of normal mouse models to investigate a human hormone. Unlike humans, mice don’t have a GH-V gene and only a pituitary version of GH is expressed during mouse pregnancy. However, similar to humans, maternal GH increases during pregnancy in mice (Gatford et al., 2017). Furthermore, mice lacking a
functional GHR have impaired reproduction (Zaczek et al., 2002). This evidence suggests that maternal GH is also important for mouse pregnancy, as it is in humans. Although the effects of human GH-V on mice have been partial elucidated by this thesis and previous studies, conclusions should be drawn cautiously. In mice and human, a complex neuroendocrine system maintains homeostasis, regulating reproduction, metabolism, energy utilization and blood pressure. When an exogenous hormone was induced to the body, the homeostasis of the neuroendocrine system was compromised. It is commonly believed that exogenous GH treatment suppresses endogenous GH secretion. In addition, other hormones in the circulation, for example PRL and glucocorticoids, are also altered. Whether the outcomes observed in GH treated animals reflect the direct effects of GH or some off-target effects due to an aberrant neuroendocrine system are not clear. Indeed it has been difficult to provide a conclusive demonstration of the physiological role of GH-V during pregnancy because of the limitations of the various experimental models. Hypophysectomized animals have a wide array of hormonal deficits; hereditary dwarf mice are deficient in GH, PRL, and thyroid-stimulating hormone (TSH), thus making separation of their effects difficult; mutant dwarf rats are not completely GH deficient (Charlton et al., 1988); and immunoneutralization of GH can provide useful information (Chandrashekar and Bartke, 1998), but is difficult to achieve, maintain for prolonged periods of time, and to convincingly demonstrate that it is complete.

The results from this thesis provide a foundation upon which a number of other studies would logically follow. The first would be to examine the effects of GH-V in isolated GH-deficient models, to control for the effects from endogenous GH. In addition, although extensive work has been done characterising the signalling pathways activated by GH-N, the signalling pathways which are activated by GH-V have not been
characterized. GH binding to its membrane-bound receptor enhances binding of JAK2 to the GHR, activates JAK2, and stimulates tyrosyl phosphorylation of both JAK2 and GHR. The activated JAK2/GHR complex recruits a variety of signalling proteins, thereby initiating multiple signalling pathways and cellular responses. These proteins and pathways include: (1) STAT transcription factors implicated in the expression of multiple genes, including the gene encoding IGF-1; (2) Shc adapter proteins that lead to activation of the grb2-SOS-Ras-Raf-MEK-ERK pathway; (3) IRS proteins implicated in the PI3K and AKT pathway; (4) signal regulatory protein α, a transmembrane scaffold protein that recruits proteins including the tyrosine phosphatase SHP2; and (5) Src homology 2 B adaptor protein 1 (SH2B1), a scaffold protein that can activate JAK2 and enhance GH regulation of the actin cytoskeleton (Carter-Su et al., 2016). SH2B1 is a newly identified key regulator of body weight and glucose metabolism (Morris and Rui, 2009). Recent studies also support a role for SH2B1 as a positive regulator of JAK2 signalling pathways initiated by leptin and, potentially, GH, as well as of pathways initiated by insulin and, potentially, by IGF-1 (Doche et al., 2012; Maures et al., 2007; Morris et al., 2010; Su et al., 2013). Given the strong homology between GH-N and GH-V, it might be expected that GH-V activates similar pathways; however, that hasn’t been determined. A key role for GH-V in metabolism in mice is suggested by the findings from this thesis. I speculate that GH-V works through various pathways to mediate these changes, and proposed that there are independent effects of GH-V during pregnancy in terms of fetal growth and maternal metabolism. Therefore, a future experiment will be to examine these various pathways in specific pregnant animal models.

In this thesis, a sensitive, specific, and relatively low-cost GH-V assay was developed. Using this assay, the serum concentration of GH-V following exogenous GH-V
administration can be monitored in follow-up animal studies, thus providing robust
evidence of the treatment. In human studies, maternal GH-V concentrations in normal
and pathological pregnancies may be more comprehensively examined in a larger
cohort. Although I did not observe any significant effect of GH-V on the tone of
uterine arteries in mice, further studies may be conducted to examine the effect of GH-
V on human arteries using placental explants. In addition, the potential mechanisms
underlying the effect of GH-V on placentation will be investigated in subsequent studies
using cell lines and primary trophoblast cells.

In summary, the work described in this thesis, using mice models and human blood
samples, has investigated the effect of GH-V on fetal growth and maternal metabolism
and proved in principle that maternal GH-V may play a unique role in fetal growth and
maternal adaptation in pregnancy. The current findings broaden our understanding of
pregnancy-related disorders and potential biomarkers for their early detections and
subsequent early interventions. Further understanding of the mechanisms in relation to
the role of GH-V during pregnancy remains the main focus of future research in this
area.
List of references


Baumbach, W.R., and Bingham, B. (1995). One class of growth hormone (GH) receptor and binding protein messenger ribonucleic acid in rat liver, GHR1, is sexually dimorphic and regulated by GH. Endocrinology 136, 749-760.


stillbirth in high-income countries: a systematic review and meta-analysis. Lancet 377, 1331-1340.


and growth hormone receptor (GHR) expression by growth hormone (GH) in mouse liver. Growth Horm IGF Res 17, 104-112.


Juul, A., Main, K., Blum, W.F., Lindholm, J., Ranke, M.B., and Skakkebaek, N.E. (1994). The ratio between serum levels of insulin-like growth factor (IGF)-I and the IGF binding proteins (IGFBP-1, 2 and 3) decreases with age in healthy adults and is increased in acromegalic patients. Clin Endocrinol (Oxf) 41, 85-93.


improves insulin sensitivity, promotes nitric oxide production, lowers blood pressure, and protects against atherosclerosis. Diabetes 61, 915-924.


(placental growth factor) and anti-angiogenic (soluble endoglin and soluble vascular endothelial growth factor receptor-1) factors in normal pregnancy and patients destined to develop preeclampsia and deliver a small for gestational age neonate. J Matern Fetal Neonatal Med 21, 9-23.


placental growth hormone in the amniotic fluid of pregnancies affected by Down syndrome. Growth Horm IGF Res 19, 121-125.


human prolactin/growth hormone family members on angiogenesis: an efficient mechanism for the regulation of angiogenesis. Proc Natl Acad Sci U S A 96, 1246-1251.


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Appendix I

The Placental Variant of Human Growth Hormone Reduces Maternal Insulin Sensitivity in a Dose-Dependent Manner in C57BL/6J Mice

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The human placental GH variant (GH-V) is secreted continuously from the syncytiotrophoblast layer of the placenta during pregnancy and is thought to play a key role in the maternal adaptation to pregnancy. Maternal GH-V concentrations are closely related to fetal growth in humans. GH-V has also been proposed as a potential candidate to mediate insulin resistance observed later in pregnancy. To determine the effect of maternal GH-V administration on maternal and fetal growth and metabolic outcomes during pregnancy, we examined the dose-response relationship for GH-V administration in a mouse model of normal pregnancy. Pregnant C57BL/6J mice were randomized to receive vehicle or GH-V (0.25, 1, 2, or 5 mg/kg · d) by osmotic pump from gestational days 12.5 to 18.5. Fetal linear growth was slightly reduced in the 5 mg/kg dose compared with vehicle and the 0.25 mg/kg groups, respectively, whereas placental weight was not affected. GH-V treatment did not affect maternal body weights or food intake. However, treatment with 5 mg/kg · d significantly increased maternal fasting plasma insulin concentrations with impaired insulin sensitivity observed at day 18.5 as assessed by homeostasis model assessment. At 5 mg/kg · d, there was also an increase in maternal hepatic GH receptor/binding protein (Ghr/Ghbp) and IGF binding protein 3 (Igfbp3) mRNA levels, but GH-V did not alter maternal plasma IGF-1 concentrations or hepatic Igf-1 mRNA expression. Our findings suggest that at higher doses, GH-V treatment can cause hyperinsulinemia and is a likely mediator of the insulin resistance associated with late pregnancy. (Endocrinology 157: 1175–1186, 2016)
creases from week 5, gradually replacing GH-N completely at approximately 20 weeks (3). The increase in maternal circulating GH-V is positively associated with fetal growth and circulating IGF-1 concentrations during pregnancy (8–12). A growth-promoting effect for GH-V has been demonstrated in vivo in nonpregnant hypophysectomized rats treated with GH-V and transgenic mice (7, 13, 14). Moreover, GH-V has proangiogenic properties (15) and stimulates trophoblast invasion in vitro and may therefore play a role in the process of placentation (16, 17).

One of the characteristic features of the maternal adaptation to pregnancy is insulin resistance with resultant hyperinsulinemia (18). This environment ensures adequate nutrient supply to the fetus. However, increased insulin resistance can lead to gestational diabetes. Placental hormones, and to a lesser extent increased fat deposition during pregnancy, may contribute to insulin resistance during pregnancy (19, 20). Consistent with this, higher concentrations of circulating GH-V have been observed in pregnancies complicated by diabetes (9, 21). Furthermore, GH-V has been demonstrated to induce severe insulin resistance and alter body composition in nonpregnant transgenic mice that overexpress GH-V (14).

Despite a proposed role for GH-V during pregnancy, the effects of GH-V administration on metabolic parameters and outcomes related to maternal and fetal growth are poorly understood. In the present study, we investigated activity of GH-V in human and mouse cell lines and examined the dose-response relationship for recombinant GH-V administration in a mouse model of normal pregnancy.

Materials and Methods

Cell lines and materials

The human prostate carcinoma cell line, LNCaP, and mouse myoblast cell line, C2C12, were obtained from the American Type Culture Collection. LNCaP cells have previously been demonstrated to only express very low levels of PRL receptor mRNA (23, 24). Cells were cultured at 37°C, 5% CO₂ in RPMI 1640 (LNCaP) or high-glucose DMEM (C2C12) (Gibco), supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and Glutamax (Gibco).

Recombinant human GH-V (22 kDa) was purchased from the Protein Laboratories Rehovot and was reconstituted in 0.4% NaHCO₃ (pH 9) (25). Recombinant human GH-N (22 kDa) was obtained from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, California).

Animals

All protocols were approved by the Animal Ethics Committee of the University of Auckland. Female C57BL/6J (B6) mice aged 8–10 weeks (Jackson Laboratories) were housed under standard conditions and maintained at 22°C with a 12-hour light, 12-hour dark cycle and with ad libitum access to food and water. Females were mated nightly with males and the day a vaginal plug detected was designated gestational day (GD) 0.5. Maternal body weight and food intake were monitored daily. At GD 12.5, pregnant mice (n = 6–7 per group) were randomized to receive GH-V (0.25, 1, 2, or 5 mg/kg · d, calculated on the basis of maternal body weight at GD 11.5) or vehicle for 6 days by osmotic pump (Alzet model 1007D; Durect Corp) inserted on the animals back, slightly posterior to the scapulae. Maternal blood was obtained via tail tip at GD 12.5 and GD 15.5. At GD 18.5, pregnant mice were fasted for 6 hours and euthanized by cervical dislocation; blood was collected by cardiac puncture. Glucose measurements were performed with a Freestyle Optium glucometer (Abbott).

Maternal tissues, fetal and placental measurement

Maternal tissues, pups, and placentas were dissected after euthanasia. Embryonic death was determined by the presence of fetal resorption, which appeared as dark round masses between live fetuses. The embryo resorption rate was calculated as the number of reabsorbed embryos/total number of embryos of each group. Maternal liver, kidneys, spleen, pancreas, perirenal fat, retroperitoneal fat and gonadal fat weights, pup weights, and placenta weights were recorded. Fetal crown-to-rump lengths and abdominal circumference were measured.

Plasma analysis

Plasma IGF-1 (Mediagnost) and insulin (CrystalChem) was assayed with a mouse-specific ELISA as per the manufacturers’ instructions. The homeostasis model assessment of insulin resistance was calculated as follows: fasting glucose (millimoles per liter) × fasting insulin (milliunits per liter)/22.5 (26).

Quantitative real-time PCR

Total RNA was isolated from liver samples using Trizol (Life Technologies). The quantity and integrity of RNA determined using a NanoDrop spectrophotometer (NanoDrop Technologies) and an Agilent Bioanalyzer RNA 6000 Nano kit, respectively. RNA integrity numbers ranged from 7.6 to 8.4. Isolated RNA was deoxyribonuclease I treated (Life Technologies). Single-stranded cDNA was synthesized from 1 µg of RNA using a Transcriptor first-strand cDNA synthesis kit (Roche), according to the manufacturer’s protocol. Real-time PCR analysis was carried out using predesigned PrimeTime qPCR assays (Integrated DNA Technologies) on a Lightcycler 480 (Roche). mRNA levels were normalized to three housekeeping genes (Gapdh, β-actin, and Cox4i1) by subtracting the geometric mean cycle threshold (Ct) of housekeeping genes from the Ct for the gene of interest to produce a ΔCt value. The ΔCt for each treatment sample was compared with the mean ΔCt for vehicle-treated samples using the relative quantification 2⁻Î”ΔCt method to determine fold change (27).

Western blotting

Cells were grown to 70%–80% confluence, serum starved for 16 hours, and treated with 500 nM GH-N or GH-V for 10 minutes, prior to lysis in 50 mM Tris-HCL (pH 7.4), 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, cOmplete protease in-
hibitor tablet (Roche), and SDS-PAGE. Where indicated, cells were treated with the human GHR antagonist (B2036, 500 nM) for 30 minutes, prior to GH-V or GH-N treatment. B2036 is the protein component of pegvisomant, a mutated human GH molecule that binds the GHR but does not activate it (28, 29). Western blot analysis was carried out under reducing conditions using a phospho-STAT5 (pTyr694) antibody (Life Technologies; 71–6900), STAT5 (C-17) antibody (Santa Cruz Biotechnology; sc-835) or mouse β-actin monoclonal antibody (Sigma-Aldrich; A1978) (Table 1). Proteins were visualized using horseradish peroxidase-conjugated secondary antibody with enhanced chemiluminescence on a BioRad Chemidoc MP system.

**Statistical analysis**

All normally distributed data are expressed as means ± SEM and were compared using a Student’s t test, a one-way ANOVA with post hoc analysis (Tukey’s procedure), or a regression analysis as appropriate. Maternal body weight and food intake data were analyzed by a repeated-measures ANOVA. ANOVA analysis and regression analysis were conducted using SigmaPlot 12.0 and IBM SPSS Statistics 21, respectively. Linear and quadratic comparisons were made among doses. A value of \( P < .05 \) was accepted as statistically significant.

**Results**

**Activation of the mouse GHR by GH-V**

To confirm activity of the recombinant human (rh) GH-V used in this study, against the human and mouse GHR receptor, activation of signal transducer and activator of transcription-5 (STAT5) signal transduction was determined in human and mouse cell lines by Western blotting. Both GH-N and GH-V stimulated STAT5 phosphorylation in the human prostate cancer cell line, LNCaP (Figure 1A). GH can activate both the GH and PRL receptors. To determine whether GH-V activation of STAT5 occurred through binding to the GHR, we investigated PRL receptor expression. We were unable to detect PRL receptor expression in LNCaP cells by semiquantitative RT-PCR (Supplemental Figure 1). Furthermore, the induction of STAT5 phosphorylation by GH-N and GH-V was abrogated by the specific GHR antagonist, B2036, thus confirming that phosphorylation of STAT5 occurred through the activation of the GHR (Figure 1A).

Figure 1. A and B, Serum-starved LNCaP cells and C2C12 cells were treated with 500 nM B2036 for 30 minutes, followed by 500 ng/mL GH-N or GH-V for 10 minutes. Immunoblotting of cell lysates for phosphorylated STAT5 (pSTAT5) and total STAT5 was performed. C, Serum-starved C2C12 cells were treated with the indicated concentrations of GH-V and GH-N for 10 minutes. Immunoblotting of cell lysates for pSTAT5 and total STAT5 was performed. β-Actin was assayed as a loading control.

**Table 1. Antibody Table**

<table>
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<tr>
<th>Peptide/Protein Target</th>
<th>Antigen Sequence (if Known)</th>
<th>Name of Antibody</th>
<th>Manufacturer, Catalog Number, and/or Name of Individual Providing the Antibody</th>
<th>Species Raised (Monoclonal or Polyclonal)</th>
<th>Dilution Used</th>
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<tr>
<td>Phospho-STAT5</td>
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<td>STAT5</td>
<td>Stat5 antibody (C-17): sc-835</td>
<td></td>
<td>Santa Cruz Biotechnology, sc-835</td>
<td>Rabbit, polyclonal</td>
<td>1:1000</td>
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<tr>
<td>β-Actin</td>
<td>Monoclonal anti-β-actin antibody produced in mouse</td>
<td></td>
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</table>

Activation of the mouse GHR by rh GH-V was confirmed in the mouse myoblast cell line, C2C12. Ghr and Prl receptor expression was detectable in C2C12 cells by semiquantitative RT-PCR (Supplemental Figure 1). Treatment with either GH-V or GH-N stimulated STAT5 phosphorylation in C2C12 cells (Figure 1, B and C). B2036 treatment reduced STAT5 activation by either GH-V or
Maternal body weight and food intake

There was no statistically significant difference in maternal body weight at the time of mating or before osmotic pump implantation. Maternal body weight increased markedly with increasing gestational age in all groups (Figure 2A). However, there was no statistically significant difference in maternal body weight and food intake between the vehicle control and GH-V treatment groups (Figure 2, A and B). A transient reduction in the maternal food intake was seen in each group after the osmotic pump implantation (Figure 2B).

Fetal growth and placental weight

There was no statistically significant difference in average litter size in each group (Table 2). Pup weight, fetal-abdominal circumference, and placental weight as well as fetal/placental ratio were not significantly different at GD 18.5 (Figure 3, A, B, and D, and Table 2). Interestingly, a small decrease in fetal crown-to rump length was observed in the 5-mg/kg GH-V treatment group, when compared with the vehicle and 0.25-mg/kg treatment groups (29.51 ± 0.15 vs 28.73 ± 0.21, P < .05 and 29.52 ± 0.13 vs 28.73 ± 0.21, P < .05, respectively) (Figure 3C). Embryonic mortality was mostly unchanged by GH-V treatment, although an increase in embryo resorption rate (6.56%) was observed in the 5-mg/kg GH-V treatment group (Table 2).

Maternal tissue weights

GH-V treatment did not affect the weights of maternal liver, kidneys, spleen, or pancreas (Table 3). There were no significant differences in maternal adipose tissue weights across all treatment groups; however, we observed a significant dose effect of GH-V on perirenal fat weight (linear, P < .05; quadratic, P < .05), with an increase in perirenal fat weight associated with increasing GH-V dose (Figure 4A). A similar significant association with dose was observed for gonadal fat weight (linear, not significant; quadratic, P < .05) (Figure 4C). These results suggest that increased GH-V during pregnancy is associated with an increase in maternal adipose deposition.

IGF-1, fasting glucose, and insulin levels

Maternal IGF-1 increased during midpregnancy and decreased in late pregnancy in all treatment groups (Table 3). However, GH-V treatment did not affect maternal IGF-1 plasma concentrations at either GD 15.5 or GD 18.5 (Table 3). Maternal fasting insulin levels were significantly increased and insulin sensitivity decreased in the 5-mg/kg treatment group at GD 18.5 (Figure 5, A and B). A dose-dependent decrease in insulin sensitivity was observed (linear, P < .01; quadratic, P < .05) (Figure 5C). No affect was seen on fasting glucose levels (Table 3).

Hepatic mRNA expression

The effect of GH-V on hepatic mRNA expression was analyzed by comparing gene expression in the vehicle-treated and 5-mg/kg GH-V treatment group (Figure 6). Hepatic Ghr/Ghbp and IGF-binding protein 3 (Igfbp3) mRNA levels were significantly increased in the 5-mg/kg treatment group (vehicle, 1.30 ± 0.16 vs GH-V, 5 mg/kg,
Table 2. Fetal and Placental Measurements

<table>
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<th>Parameters</th>
<th>Vehicle (n = 7)</th>
<th>0.25 mg/kg (n = 6)</th>
<th>1 mg/kg (n = 6)</th>
<th>2 mg/kg (n = 6)</th>
<th>5 mg/kg (n = 7)</th>
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<tr>
<td>Litter size</td>
<td>8.7 ± 0.4</td>
<td>9.0 ± 0.6</td>
<td>9.2 ± 0.5</td>
<td>8.0 ± 0.5</td>
<td>8.0 ± 0.9</td>
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<tr>
<td>Pup weight, mg</td>
<td>1235 ± 25.6</td>
<td>1188 ± 11.2</td>
<td>1188 ± 26.7</td>
<td>1169 ± 25.6</td>
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<td>Placental weight, mg</td>
<td>85.1 ± 2.2</td>
<td>81.9 ± 1.5</td>
<td>82.2 ± 2.8</td>
<td>82.7 ± 0.7</td>
<td>86.9 ± 3.2</td>
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<tr>
<td>Fetal to placental ratio</td>
<td>14.5 ± 0.5</td>
<td>14.7 ± 0.2</td>
<td>14.7 ± 0.4</td>
<td>14.3 ± 0.3</td>
<td>13.5 ± 0.4</td>
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<td>Reabsorption rate, %</td>
<td>0</td>
<td>0</td>
<td>1.79</td>
<td>0</td>
<td>6.56</td>
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</tbody>
</table>

Data are presented as mean ± SEM (n = number of litters per group). There are no significant differences across any of the treatment groups.

* Number of reabsorbed embryos per total number of embryos in each group.

1.99 ± 0.11, P < .01, and 0.92 ± 0.07 vs 1.29 ± 0.12, P < .05, respectively). Mouse GHBP is generated through alternative splicing of RNA transcripts from the Ghr/Ghbp gene. The primers used in our study do not distinguish between these two transcripts. mRNA levels of the solute carrier family 2, member 4 (Slc2a4, Glut4) were significantly decreased after GH-V treatment (0.92 ± 0.14 vs 0.45 ± 0.08, P < .05). However, GH-V treatment did not alter the expression of hepatic insulin receptor substrate-1 (Irs-1), insulin receptor (Insr), v-akt murine thymoma viral oncogene homolog 3 (Akt3), Igf-1, IGFBP acid labile subunit (Als), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3-kinase) catalytic subunit-α (Pik3ca), or PI3-kinase regulatory subunit-α (Pik3r1).

### Discussion

Recombinant GH-N therapy has long been used as an effective treatment for promoting growth due to its somatotrophic properties. However, GH-N treatment can induce insulin resistance, edema, and alterations in carbohydrate and lipid metabolism (30). GH-V is secreted from the placenta during human pregnancy and may also be associated with fetal growth in humans (8–12). Previous studies have observed growth-promoting properties of 22 kDa GH-V in rodents (7, 13, 14, 31). However, these studies were conducted in nonpregnant animals. The aim of the current study was to evaluate the physiological effects of GH-V administration in a mouse model of pregnancy.

Despite previous reports of growth-promoting effects in nonpregnant mice, we did not observe any difference in maternal or fetal weight with increasing GH-V dose, although the fetal crown-rump length was reduced in the 5-mg/kg treatment group. This is consistent with work by Naar et al (32), who observed reduced fertility with compromised fetal growth in transgenic mice overexpressing human GH-V. Other studies have investigated the effect of maternal GH treatment on fetal growth during pregnancy with variable outcomes. Zamenhof et al (33) treated pregnant rats with bovine GH from day 7 to day 20 of pregnancy, with no change in fetal weight but a significant increase in brain weight. Gargosky et al (34) treated pregnant rats with rhGH-N or human IGFL-1 via an osmotic pump, but neither treatment affected fetal or placental weights. In sheep, Jenkin-
son et al (35) treated pregnant ewes with bovine GH during different stages of gestation and found that it stimulated fetal growth only after day 100 of gestation, whereas Harding et al (36) found that neither fetal or maternal growth was altered by bovine GH treatment from day 125 to day 134 of gestation. Discordant results on fetal growth have also been seen in pigs after GH treatment (37–40). It is likely that different GH preparations, dose regimens, and treating periods may contribute to these findings. Moreover, nutrient partitioning may also play an important part in fetal growth (38, 41, 42), and it has been

Figure 4. Maternal adipose tissue weights. A, Perirenal fat. B, Retroperitoneal fat. C, Gonadal fat. There were significant dose effects of GH-V on maternal perirenal and gonadal fat weights using linear and quadratic regression analysis. Bars indicate mean values. Veh, vehicle.

Figure 5. Maternal plasma insulin levels and homeostatic model assessment (HOMA) of insulin sensitivity. A, Maternal fasting insulin levels at GD 18.5. B and C, HOMA for estimation of insulin resistance (IR) and sensitivity (%S) was determined using the HOMA2 Calculator version 2.2.3 (Diabetes Trials Unit, University of Oxford. Oxford, United Kingdom). A and B, Data are presented as mean ± SEM. C, Bars indicate mean values. Groups that do not share the same letter are significantly different from each other. There was a dose-dependent decrease in insulin sensitivity using linear and quadratic regression analysis, n = 6 (0.25, 1, 2 mg/kg · d) or n = 7 (vehicle, 5 mg/kg · d) per group. Veh, vehicle.
suggested that the anabolic effect of exogenous GH on the mother may counteract the growth-promoting effect of GH treatment on the fetus by reducing the nutrient supply (36, 43).

Previous studies have demonstrated that placental lactogen is responsible for the maintenance of pregnancy and a series of actions include promotion of fetal growth in mice (44–46). Because human GH exhibits lactogenic activity in rodents (47, 48), it has been hypothesized that human GH administration may interfere with endogenous lactogen release in the rodent or that high levels of GH act as an antagonist at the lactogen receptors but exhibit insufficient lactogenic effects during pregnancy (32, 49). Maternal glucocorticoid levels may also be involved in the effect of GH treatment on fetal growth. Increased maternal glucocorticoid levels impair fetal growth during pregnancy (50–53). Because elevated glucocorticoid levels are observed in transgenic mice overexpressing the human or bovine GH gene (54, 55), maternal glucocorticoids may play a role in fetal growth after GH administration, although the chronic GH effects in transgenic mice may not be comparable with the relatively acute effects of GH administration during pregnancy. In fact, GH administration during pregnancy may elicit a number of interacting effects across the entire neuroendocrine systems. In our study, GH-V treatment did not promote maternal or fetal body weight but impaired fetal linear growth. Other possible mechanisms cannot be excluded (Table 2).

Surprisingly, we observed a trend of increased maternal adipose tissue weight with increasing doses of GH-V. Although GH is widely recognized to have lipolytic properties, conflicting reports exist, and it has been claimed that GH-N interacts with adipose tissue in different ways to promote both lipolytic and antilipolytic effects (31, 56, 57). In in vivo studies, it has been shown that GH-N administration reduces lipolysis and free fatty acids in both humans and animals (58–61), although this effect is transient and is observed only in the early period after GH-N injections with subsequent lipolytic effects (62–64). In addition, in some studies young GH transgenic mice (≤6 mo of age) show increases in fat mass, whereas a reduction in adipose mass is observed in older mice (65). GH-N and GH-V share similar structures and physiological effects. However, whether GH-V has similar actions on adipose tissue is largely unknown, especially during pregnancy, and the exact effects of GH-V on maternal adipose tissue remain unclear.

We observed that GH-V treatment significantly increased maternal fasting plasma insulin concentrations and insulin resistance on GD 18.5 at the 5-mg/kg dose, with no corresponding changes in fasting glucose concentrations. After conditions of GH deprivation, exposure to GH-N elicits short-term insulin-like effects, whereas chronic GH-N leads to well-documented antagonistic effects on insulin action and consequently insulin resistance (66, 67). The GH-N and insulin receptor signaling pathways share several downstream signaling components. Similar to the action of insulin, GH-N induces tyrosyl phosphorylation of IRS proteins, providing binding sites for the regulatory subunit (p85) of PI3-kinase (67, 68).
GH-N activation of PI3-kinase plays an important role in glucose transport and lipid synthesis (67, 68). However, chronic GH-N leads to decreased IRS-1 in skeletal muscle and reduced tyrosine phosphorylation of IRS-1 and stimulates serine phosphorylation of IRS-1, which inhibits the interaction of IRS-1 with the insulin receptor (66, 69). GH also increases levels of p85, which competes with p85-p110 heterodimers for binding to IRS-1 and negatively regulates PI3-kinase signaling (70). Studies investigating the effect of GH-V on insulin resistance are more limited. However, a series of studies investigating insulin resistance in transgenic mice (14, 71, 72) demonstrated that GH-V does not affect phosphorylation of IRS-1 in the skeletal muscle of transgenic mice (71) but causes insulin resistance by specifically increasing the protein expression of the p85 subunit of PI3-kinase in muscle and adipose tissue and subsequently reducing PI3-kinase signaling (71, 72) (Table 3).

In our study, the mRNA expression levels of hepatic Irs-1, Insr, Akt3, Pik3ca, and Pik3r1 were unaltered after GH-V treatment. However, hepatic expression of the gene for insulin-sensitive glucose transporter 4 (Slc2a4/Slut4) was significantly down-regulated. Reduced expression of Glut4 has been associated with insulin resistance and plays a role in the pathophysiology of type 2 diabetes mellitus (73). This may contribute to the insulin resistance induced by GH-V. Altered mRNA or protein expression of Irs-1, Insr, Akt3, Pik3ca, and Pik3r1 may occur in other tissues and is under investigation.

IGF-1 is a primary mediator of the effects of GH, in particular its growth-promoting effects. Circulating IGF-1 is synthesized mainly by the liver under the control of GH. The binding of GH with its hepatic receptor stimulates expression and release of IGF-1 into the circulation (73). This may contribute to the insulin resistance induced by GH-V. Altered mRNA or protein expression of Irs-1, Insr, Akt3, Pik3ca, and Pik3r1 may occur in other tissues and is under investigation.

Recent studies suggest that IGF-1 may not be a good biomarker for GH administration in the normal mouse, despite human GH showing bioactivity in rodents. Bielohuby et al (86) compared IGF-1 responsiveness in different mouse strains after mouse, bovine, and human pituitary GH administration and found no significant increase in IGF-1. However, GH was found to be active, increasing body weight and organ size, and activating STAT5 in the liver. The authors speculate that IGF-1 release is already maximal in these animals and cannot be further increased by exogenous GH treatment (86).

### Table 3. Maternal Tissue Weights and Plasma Glucose and IGF-1 Concentrations

<table>
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<tr>
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</tr>
</thead>
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<tr>
<td>Liver weight, mg</td>
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<tr>
<td>Kidney weight, mg</td>
<td>139.0 ± 4.4</td>
</tr>
<tr>
<td>Spleen weight, mg</td>
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</tr>
<tr>
<td>Pancreas weight, mg</td>
<td>213.9 ± 12.6</td>
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<tr>
<td>Fed glucose, mmol/L</td>
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</tr>
<tr>
<td>Fasting glucose, mmol/L</td>
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</tr>
<tr>
<td>IGF-1 at GD 12.5, ng/mL</td>
<td>325.7 ± 21.4</td>
</tr>
<tr>
<td>IGF-1 at GD 15.5, ng/mL</td>
<td>308.0 ± 10.6</td>
</tr>
<tr>
<td>IGF-1 at GD 18.5, ng/mL</td>
<td>244.5 ± 33.6</td>
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</table>

Data are presented as mean ± SEM. There are no significant differences across any of the treatment groups.

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Most circulating IGF-1 in humans and mice is bound to IGFBP3 and ALS to form a 150-kDa ternary complex and is the major storage form of IGF-1 in the circulation (87). This complex prolongs the half-life of circulating IGF-1 and facilitates its endocrine actions. IGFBP3 has been shown to either inhibit or augment IGF-1 actions. Overexpressing IGFBP3 in mice resulted in intrauterine fetal growth retardation despite elevated circulating IGF-1 concentrations (88). On the other hand, both IGF-1 and IGFBP3 are elevated concordantly in acromegaly patients, yet there is no growth inhibition due to the equilibrium between IGF-1 and IGFBP3 concentrations (89). The liver is believed to be the main source of IGF-1, IGFBP3, and ALS in the circulation (90). We observed that hepatic Igfbp3 mRNA levels were significant increased after GH-V treatment. In addition, there was a trend for increased Als mRNA expression, whereas maternal plasma IGF-1 concentrations were unaltered. Increased IGFBP3 may diminish free IGF-1 in the maternal circulation and thus reduce the bioactivity of IGF-1. This may partially explain the reduced fetal length observed in our study.

We found that GH-V increased liver Ghr/Ghbp expression during mouse pregnancy. This is consistent with studies using GH in pregnant mice (83) and rats (91). Jiang et al (92) found that GH increased hepatic Ghr expression in a bovine model. However, Mathews et al (93) found no significant changes in hepatic Ghr mRNA levels between control and hypophysectomized rats treated with bovine GH, although pregnant females had elevated Ghr expression. The time, dose, and duration of GH exposure, in vivo or in vitro experiments, steroid hormones, and nutritional status may all contribute to the variation shown across models (94).

In contrast to the situation in humans, mouse GHBP is generated through alternative splicing of RNA transcripts from the Ghr/Ghbp gene (95). Circulating concentrations of pituitary GH increase during mouse pregnancy. Expression of the mouse GHR and GHBP also increase substantially. It is therefore thought that the concentration of free circulating GH may remain unchanged during mouse pregnancy (83, 95). Early studies demonstrated that mouse liver GHBP predominantly exists as a membrane-associated protein, which is structurally distinct from the soluble form of GHBP present in serum. It has been suggested that membrane-associated GHBP may function as a cell-surface receptor for GH in the liver through interaction with integrins (95, 96). However, an alternative explanation is that membrane-associated GHBP may act to attenuate the effects of high concentrations of GH (97).

There are other differences in the GH axis in mouse and human pregnancy, which should be highlighted with regard to our study. As described above, the human GH gene family is a cluster of five genes, which includes GH-N, GH-V, and the chorionic somatomammotropin (CS-A, CS-B, and CS-L) genes (98). In contrast, the rodent genome contains a single pituitary-specific GH gene and lacks any GH-related chorionic somatomammotropin genes (99). Consequently, only a pituitary version of GH is expressed during rodent pregnancy (83, 95). It is not clear why the primate locus has evolved independently to encompass a pituitary and placental version of the GH gene. Despite these differences, extrapituitary expression of GH is observed in multiple tissues in both humans and mice, including the mouse placenta (100), which suggests a potential role in mouse pregnancy. So although the mice lack a GH-V gene, the mouse is still a useful model to determine effects of GH-V. Further studies would be required to determine whether there are differences in the action of GH-N vs GH-V or whether the mode of presentation of GH (pulsatile vs tonic) may be more important.

Although human GH-N and GH-V can both bind and activate the GHR of nonprimate species, we cannot exclude species-specific differences.

In conclusion, higher doses of GH-V-induced hyperinsulinemia in pregnant C57BL/6J mice, suggesting that GH-V is a likely candidate to induce insulin resistance during pregnancy. Although GH-V treatment did not promote fetal growth in our studies, due to the intimate relationship between GH-V and fetal growth during human pregnancies, further investigation of specific animal models, particularly those of growth restriction, are warranted.

Acknowledgments

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Disclosure Summary: The authors have nothing to disclose.

References

3. Eriksson L, Frankenne F, Eden S, Hennen G, Vonschoul B. Growth-hormone 24-h serum profiles during pregnancy—lack of


37. Gutfreund KI, Querelle JT, Heinemann GK, et al. Increased placental nutrient transporter expression at midgestation after maternal...
82. Vickers MH, Gilmour S, Gertler A, et al. 20-kDa placental hGH-V has diminished diabetogenic and lactogenic activities compared


Appendix II

Comparison of pulsatile vs. continuous administration of human placental growth hormone in female C57BL/6J mice

Shutan Liao1,2,3 · Mark H Vickers1,2 · Angharad Evans1 · Joanna L Stanley1,2 · Philip N Baker1,2 · Jo K Perry1,2

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Abstract Exogenous growth hormone has different actions depending on the method of administration. However, the effects of different modes of administration of the placental variant of growth hormone on growth, body composition and glucose metabolism have not been investigated. In this study, we examined the effect of pulsatile vs. continuous administration of recombinant variant of growth hormone in a normal mouse model. Female C57BL/6J mice were randomized to receive vehicle or variant of growth hormone (2 or 5 mg/kg per day) by daily subcutaneous injection (pulsatile) or osmotic pump for 6 days. Pulsatile treatment with 2 and 5 mg/kg per day significantly increased body weight. There was also an increase in liver, kidney and spleen weight via pulsatile treatment, whereas continuous treatment did not affect body weight or organ size. Pulsatile treatment with 5 mg/kg per day significantly increased fasting plasma insulin concentration, whereas with continuous treatment, fasting insulin concentration was not significantly different from the vehicle-treated control. However, a dose-dependent increase in fasting insulin concentration and decrease in insulin sensitivity, as assessed by HOMA, was observed with both modes of treatment. At 5 mg/kg per day, hepatic growth hormone receptor expression was increased compared to vehicle-treated animals, by both modes of administration. Pulsatile variant of growth hormone did not alter the plasma insulin-like growth factor-1 concentration, whereas a slight decrease was observed with continuous variant of growth hormone treatment. Neither pulsatile nor continuous treatment affected hepatic insulin-like growth factor-1 mRNA expression. Our findings suggest that pulsatile variant of growth hormone treatment was more effective in stimulating growth but caused marked hyperinsulinemia in mice.

Keywords Placental growth hormone · Delivery methods · Insulin sensitivity · Mice

Introduction

The growth hormone (GH) and insulin-like growth factor-1 (IGF-1) axis is a major regulator of mammalian growth, reproduction and cell regeneration. In humans, two GH genes encode two GH proteins: pituitary GH (GH-N, GH1) and placental GH variant (GH-V, GH2) [1]. The protein sequences of GH-N and GH-V are highly conserved, differing by 13 of 191 amino acids [2]; however, GH-V has an N-glycosylation site and is more basic in structure than GH-N [3]. In addition, they have distinct expression profiles; GH-N is mainly secreted in a pulsatile fashion from the pituitary, while GH-V is secreted from the placenta in a non-pulsatile manner during human pregnancy. The continuous secretion of GH-V into the maternal compartment is thought to contribute to maternal metabolic alterations during pregnancy [4]. Both proteins bind the GH receptor (GHR) with similar affinity and share similar physiological somatotrophic, lactogenic and lipolytic properties [5, 6]. However, compared with GH-N, GH-V binds the prolactin (PRL) receptor poorly and its lactogenic effects are greatly reduced [7, 8]. GH-V replaces GH-N as the dominant
circulating form of GH at approximately 20 weeks of gestation [4]. The increase in maternal circulating GH-V is positively associated with foetal growth and circulating IGF-1 concentrations during pregnancy [9–13].

The effects of exogenous GH-N on growth, body composition and carbohydrate metabolism are well documented [9] and it has been noted that different effects are observed in response to different modes of delivery. Pulsatile infusion of human or bovine GH, compared to continuous infusion, is more effective in stimulating growth in hypophysectomized rats [14, 15]. Differences in growth rate and body composition were also observed in intact rats [16, 17]. In addition, GH-N has different effects on glucose homoeostasis and lipid profiles in GH-deficient adults depending on whether administration is by daily injection or continuous infusion [18].

Although GH-V has been demonstrated to stimulate growth, alter body composition and induce insulin resistance (IR) in hypophysectomized rats and transgenic mice [8, 19, 20], there is relatively little information on the effects of GH-V utilizing different modes of administration. The aim of the current study was to determine the effect of delivery methods on growth and metabolic outcomes by examining the effect of pulsatile vs. continuous administration of recombinant GH-V in a normal mouse model.

Materials and methods

Materials

Mouse BA/F3 cells stably expressing the human GHR (BA/F3-GHR) were a kind gift from Professor Mike Waters (University of Queensland, Australia). Cells were cultured at 37 °C, 5% CO2 in RPMI (Gibco) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and Glutamax (Gibco). BA/F3 cells were cultured in the presence of 50 ng/ml GH and 10 ng/ml interleukin-3.

Recombinant human GH-V (22 kDa) was purchased from Protein Laboratories Rehovot (Rehovot, Israel) and was reconstituted in 0.4% NaHCO3 pH 9 [21].

Animals

All protocols were approved by the Animal Ethics Committee of the University of Auckland. Female C57BL/6J (B6) mice aged 6–8 weeks (Jackson Laboratories) were housed under standard conditions and maintained at 22 °C with a 12 h light/dark cycle and with ad-libitum access to food and water. Mice were housed in pairs. A total of 36 mice, averaging 19.5 ± 0.1 g initial body weight, were assigned to treatment groups (n = 6 per group as detailed below). Mean body weight and weight range within each group was the same at the start of treatment.

Treatments

Mice were randomized to receive GH-V (2 or 5 mg/kg per day; calculated on the basis of pretreatment body weight) or vehicle for 6 days by either subcutaneous injection (SC) or osmotic pump (OP) (Alzet model 1007D, Durect Corporation, Cupertino, CA).

For SC groups, GH-V or vehicle (100 µl) was administered subcutaneously in the skinfold at the nape of the neck using tuberculin syringes twice a day (8 am and 5 pm) from day 1 to day 6 with the last injection occurring on day 7 at 8 am. Lyophilized powder was solubilized to the target concentration prior to use on each treatment day.

For OP groups, pump selection was based on the size, duration and flow rate. Model 1007D (100 µl) was designed to release its contents at a rate of 0.5 µl/h over 1 week duration. Pumps were filled with reconstituted recombinant human GH-V or vehicle and placed in sterile 0.9% saline at 37 °C for priming overnight. On day 1, pumps were inserted on the back of animal, slightly posterior to the scapulae. On day 4, a blood sample was obtained via tail tip from the OP groups. Delivery was verified by measurement of the residual volume in the pump reservoir after removal on day 7.

Body weights and food intake were monitored daily from day 0 to day 7. On day 7, mice were fasted for 4 h from 10 am to 2 pm, and euthanized by cervical dislocation. Blood was collected by cardiac puncture and kept on ice until centrifugation and removal of supernatant for plasma analysis. Glucose measurements were performed with a Freestyle Optimum glucometer (Abbott, UK). The weights of the liver, kidneys, spleen, heart, perirenal fat, retroperitoneal fat and gonadal fat were recorded and tissues stored at −80 °C.

AlphaScreen assay

To ensure continuous delivery in the OP treatment groups, the stability of recombinant GH-V protein at 37 °C was verified using an AlphaScreen assay. 500 ng/ml GH-V solubilised in 0.4% NaHCO3 pH 9 was incubated at 37 °C for 7 days with samples taken each day and stored at −80 °C for later analysis. Dose-response assays were also carried out with unfrozen GH-V on day 0, 3 and 6 and the half maximal effective concentration (EC50) calculated. BA/F3-GHR cells were serum starved for 16 h and treated with GH-V for 10 min. An AlphaScreen SureFire p-STAT5 (PerkinElmer, USA) was used to measure the phosphorylation level of STAT5A and STAT5B in cellular lysates and was performed as per the manufacturer’s instructions. Alphascreen signal (counts) was read on an EnVision Multilabel plate reader (PerkinElmer).
Plasma analysis

Plasma IGF-1 (Mediagnost, Germany) and insulin (CrystalChem, USA) were assayed with mouse-specific enzyme-linked immunosorbent assays (ELISA) as per the manufacturer’s instructions. The homoeostasis model assessment of IR (HOMA-IR) was calculated as: Fasting glucose (mmol/l) × fasting insulin (mU/l)/22.5 [22].

Quantitative real-time PCR

Total RNA was isolated from liver samples using Trizol (Life Technologies). The quantity and integrity of RNA were determined using a NanoDrop spectrophotometer (NanoDrop Technologies) and an Agilent Bioanalyzer RNA 6000 Nano kit, respectively. RNA integrity number (RIN) ranged from 9.2 to 9.8. Isolated RNA was DNase I treated (Life Technologies). Single-stranded cDNA was synthesized from 1 μg of RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche), according to the manufacturer’s protocol. Real-time PCR analysis was carried out using predesigned PrimeTime qPCR assays (Integrated DNA Technologies) on a Lightcycler 480 (Roche). mRNA levels were normalized to three housekeeping genes: Gapdh, β-Actin and Cox4i1 by subtracting the geometric mean Ct of housekeeping genes from the Ct for the gene of interest to produce a ΔCt value. The ΔCt for each treatment sample was compared with the mean ΔCt for vehicle-treated samples using the relative quantification 2−ΔΔCt method to determine fold-change [23].

Statistical analysis

All normally distributed data are expressed as means ± S.E.M and were compared using Student’s t-test, one-way ANOVA with post-hoc analysis (Tukey’s procedure), or regression analysis as appropriate. Body weight and food intake data were analysed by repeated measures ANOVA. ANOVA analysis and regression analysis were conducted using SigmaPlot 12.0 and IBM SPSS Statistics 21, respectively. Linear comparisons were made among doses. In vitro assays were repeated at least three times with a representative figure shown. A p value of < 0.05 was accepted as statistically significant.

Results

Recombinant GH-V protein stability

The bioactivity of GH-V in OPs over the treatment period was estimated by incubating GH-V at 37 °C for 6 days and measuring the phosphorylation of STAT5 in GH-V-treated BA/F3-GHR cells using an AlphaScreen assay. A 26 % reduction in GH-V bioactivity was observed after 6 days incubation at 37 °C (Fig. 1). No statistically significant difference in bioactivity was observed from day 0–3. Dose-response curves were carried out on day 0, 3 and 6 and the EC50 measured. A small increase in the EC50 concentration was observed at day 3 (152.2 ng/ml) and day 6 (122.8 ng/ml), compared to day 0 (90.9 ng/ml), however, these differences were not significant.

Body weight and food intake

GH-V treatment with 2 and 5 mg/kg per day via SC significantly increased body weight (repeated measures ANOVA, p < 0.05) without affecting food intake, compared with vehicle-treated animals (Fig. 2a-b). There was no statistically significant difference in body weight or food intake among groups following OP treatment (Fig. 2c–d), although a transient reduction in food intake was seen following OP implantation (Fig. 2d).

Tissue weights

GH-V treatment with 5 mg/kg per day via SC significantly increased liver, kidney and spleen weights (one-way ANOVA, p < 0.05), but only liver weight was increased following 2 mg/kg per day SC treatment (one-way ANOVA, p < 0.01) (Fig. 3). GH-V treatment via OP did not affect the weight of the liver, kidney, or spleen (Fig. 3). Heart weight was not affected by different administration methods or treatment doses. There were no significant differences in adipose tissue weights across treatment groups (Fig. 4).
Plasma IGF-1, fasting glucose and insulin concentrations

In the SC groups, there was no effect of GH-V treatment on IGF-1 plasma concentration after 6 days of treatment (Fig. 5a). In the OP treatment group, GH-V treatment did not affect IGF-1 plasma concentration at day 4 (Fig. 5b). However, we observed a significant dose effect of GH-V on IGF-1 concentration (linear trend, \( p < 0.05 \)), with a decrease in IGF-1 concentration associated with increasing GH-V dose at day 7 in OP groups (Fig. 5c).

As no stimulatory effect on circulating IGF-1 was observed, we tested whether GH-V was capable of stimulating \( I gf-1 \) mRNA expression in the mouse myoblast cell line C2C12, which is known to up-regulate \( I gf-1 \) mRNA expression in response to GH-N treatment [24, 25]. Both GH-N and GH-V increased expression of \( I gf-1 \) in this cell line (data not shown).

Fasting insulin concentration was significantly increased in the 5 mg/kg SC treatment group, compared to vehicle (one-way ANOVA, \( p < 0.05 \)) (Fig. 6a). However, fasting insulin concentrations were not significantly different from the vehicle-treated control in the OP groups (Fig. 6b), a dose-dependent increase in fasting insulin concentration and decrease in insulin sensitivity was observed in both SC and OP administration treatment groups, as assessed by HOMA (linear trend, \( p < 0.05 \)) (Fig. 6a–d). No effect was seen on fasting glucose concentrations (data not shown).

Hepatic mRNA expression

The effect of GH-V on hepatic mRNA expression was analysed by comparing gene expression in the vehicle-treated and 5 mg/kg GH-V treatment group (Table 1). GH-V treatment via SC and OP significantly increased hepatic \( G hr/G hbp \) expression (\( p < 0.05 \)) (Table 1). It should be noted that mouse
Fig. 3 Tissue weights. *Bars* indicate mean values. Groups which do not share the same letter are significantly different from each other (*p* < 0.05)
GH binding protein (GHBP) is generated through alternative splicing of RNA transcripts from the Ghr gene. The primers used in our study do not distinguish between these two transcripts. mRNA expression of hepatic Igf-1 and insulin-like GHBP 3 (Igfbp3) was not affected by GH-V treatment, but IGFBP acid labile subunit (Als) expression was significantly decreased after GH-V OP treatment ($p < 0.05$). Moreover, mRNA levels of v-akt murine thymoma viral oncogene homologue 1 (Akt1) in SC group was significantly increased ($p < 0.05$), whereas expression in the OP group was not affected. Additionally, changes in insulin receptor (Insr) expression after GH-V OP treatment exhibited a marginal trend towards significance ($p = 0.051$). However, GH-V treatment did not alter the expression of hepatic insulin receptor substrate-1 (Irs-1), Akt2, Akt3, Solute carrier family 2, member 1, 2 and 4 (Glut1, 2 and 4), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3-kinase) catalytic subunit alpha (Pik3ca), or PI3-kinase regulatory subunit alpha (Pik3r1).

**Discussion**

Previous studies have found that the induction of the growth and metabolic response to GH varies according to the dose
and regulates the expression of several sex-specific phenotypes [28–31]. In females, the pulses of GH-N secretion are lower and plasma GH-N baseline level is higher than in males [29].

The effects of different modes of GH-N delivery on regulation of growth have been studied previously. Jansson et al. [32] and Thorngren et al. [33] observed that the frequency of GH administration influenced body growth in hypophysectomized rats. However, the growth response did not increase proportionally to an increased administration frequency. The “stress” associated with injections may contribute to this. To avoid frequent injections, an OP was designed to mimic the continuous fashion accumulated by multiple injections. In terms of growth stimulation, pulsatile GH administration has been inferred to be superior to continuous delivery [34]. Clark et al. treated hypophysectomised rats with recombinant human GH (0.04, 0.2, 1, or 5 mg/kg per day) for 7 days and found that growth responses depended on the pattern of GH administration (twice daily injections > continuous infusions > daily injections) [35]. Consistent with previous studies, we found that GH-V treatment via twice daily SC is more effective in stimulating growth than OP and significantly increased body weight and organ size. However, despite administration in comparable doses, GH-V treatment via OP did not affect animal growth.

The mechanism underlying this differential responsiveness to the mode of administration is still unclear. IGF-1 is a primary mediator of the effects of GH-N, in particular its growth-promoting effects. Circulating IGF-1 is synthesized mainly by the liver under the control of GH-N. The binding of GH with its hepatic receptor stimulates expression and release of IGF-1 into the circulation [36]. Moreover, it has been proposed that GH-V substitutes for GH-N to regulate maternal circulating IGF-1 concentration during pregnancy [37, 38]. Transgenic mice which overexpress human GH-V have increased circulating IGF-1 concentration [20, 39]. Maiter et al. found that GH-N administration with intermittent delivery caused higher serum IGF-1 concentration that with continuous delivery in rats [34]. In our study, GH-V treatment did not affect plasma IGF-1 concentration or hepatic Igf-1 expression via either SC or OP administration but a trend for decreasing IGF-1 concentrations was observed with increasing GH-V doses in the OP group. Of note, a recent study suggests that IGF-1 may not be a good biomarker for GH administration in wild-type mice, despite human GH showing bioactivity in rodents. Bielohuby et al. compared IGF-1 responsiveness in different mouse strains following mouse, bovine and human GH-N administration and found no significant increase in IGF-1. The authors speculate that IGF-1 release is already maximal in normal animals and cannot be further increased by exogenous GH treatment [40]. Nonetheless this does not exclude the
possibility that local generation of IGF-1 in some tissues apart from liver may play a role in the growth-promoting effect of GH. Isgaard et al. observed that pulsatile and continuous GH treatment were equally effective in stimulating hepatic Igf-1 expression in hypophysectomized rats, whereas only pulsatile GH infusion had a marked stimulatory effect in skeletal muscle [15]. Further, different patterns of GH treatment also affect GH sensitivity by altering GH binding. Continuous infusion of GH has been found to be more potent in increasing the number of GH and prolactin binding sites than injections [34, 41]. Baumbach et al. found that GH treatment by continuous infusion significantly induced Ghr expression in hypophysectomised female and castrated male rats, but a single injection did not [42]. These findings are consistent with reports of hepatic GHR and serum GHBP in dwarf rats [43, 44]. Baumbach et al. found that GH treatment by continuous infusion significantly induced Ghr expression in hypophysectomised female and castrated male rats, but a single injection did not [42]. These findings are consistent with reports of hepatic GHR and serum GHBP in dwarf rats [43, 44]. Additionally, only a small percentage of the mouse GH is bound to GHBP in normal mice. Given that the binding affinity of human GH for the murine GHR and GHBP is higher than the affinity of mouse GH [45], different circulating GH-V concentrations generated by administration patterns may have different impacts on GH-GHBP binding and effects in mice.

In the present study, we observed that GH-V treatment utilizing two modes of administration significantly increased hepatic Ghr/Ghbp expression in mice. In contrast to humans, the mouse GHR gene contains a special exon (exon 8A), which encodes a GHBP specific hydrophilic sequence [46]. Therefore, GHBP is generated through alternative splicing of RNA transcripts from the Ghr/Ghbp gene [47] and its expression clearly depends on GH concentrations [45, 48]. Early studies demonstrated that mouse liver GHBP predominantly exists as a membrane-associated protein, which is structurally distinct from the soluble form of GHBP present in serum, and the membrane-associated GHBP may function as a cell-surface receptor for GH in the liver through interaction with integrins [47, 49]. However, an alternative explanation is that membrane-associated GHBP may act to attenuate the effects of high concentrations of GH [50].

The majority of circulating IGF-1 in humans and mice is bound to IGFBP3 and ALS to form a 150 kDa ternary complex and is the major storage form of IGF-1 in the circulation [51]. ALS stabilizes the IGF-IGFBP3 complex, reduces the passage of IGF-1 to the extravascular...
However, this was not significant in the SC group there was a trend for increased expression; however, this was not significant. Surprisingly, continuous treatment of GH-V (5 mg/kg per day) induced a slight decrease in hepatic AlS mRNA expression (1.4-fold). A decrease in AlS expression may contribute to the dose-dependent decrease in IGF-1 concentration observed in OP groups, as previous studies have found that ALS depletion induces a mild growth deficit despite a large reduction in the concentration of IGF-1 [56, 57].

While GH is widely recognized to be lipolytic, conflicting reports exist and it has been claimed that GH-N interacts with adipose tissue in different ways to promote both lipolytic and anti-lipolytic effects [58, 59]. Moreover, free fatty acid concentrations increased after GH treatment in both normal individuals [60] and GH-deficient patients [61]. However, the lipolytic effect of GH-N is more pronounced via pulsatile administration [62, 63]. Although GH-V exhibits similar effects to GH-N on fat metabolism in the rat [64], adipose tissue weight was not altered by GH-V treatment in our study. The exact effects of GH-V on adipose tissue currently remain unclear.

GH is an important regulator of insulin sensitivity. Following conditions of GH deprivation, exposure to GH-N elicits short-term insulin-like effects, whereas chronic GH-N leads to well-documented antagonistic effects on insulin action, and consequently IR [65]. The GHR and insulin receptor signalling pathways share several downstream signalling components [66]. Similar to the action of insulin, GH-N induces tyrosyl phosphorylation of IRS proteins, providing binding sites for the regulatory subunit (p85) of phosphoinositide 3-kinase (PI3-kinase) [67]. GH-N activation of PI3-kinase and its downstream effector plays an important role in glucose transport and lipid synthesis [66, 68]. However, chronic excess GH-N exposure is associated with decreased response to insulin stimulation by decreasing IRS-1 in skeletal muscle, reducing tyrosine phosphorylation of IRS-1, and stimulating serine phosphorylation of IRS-1 which inhibits interaction of IRS-1 with the insulin receptor [65, 69]. GH also increases levels of p85 that competes with PI3-kinase heterodimers for binding to IRS-1 and negatively regulates PI3-kinase signalling [70]. Administration pattern of GH has been shown to influence insulin sensitivity. Higher insulin concentration was seen in continuous GH treatment in rats [16]. In GH-deficient patients, daily GH injections administered for a short period (2–4 weeks) exacerbated impaired glucose tolerance when compared to continuous infusion [62, 63], but the GH pattern did not influence insulin sensitivity after 6 months treatment [71]. Studies investigating the effect of GH-V on IR are more limited. A series of studies investigating IR in transgenic mice [20, 72, 73], demonstrated that GH-V does not affect phosphorylation of IRS-1 in the skeletal muscle of transgenic mice [72], but causes IR by specifically increasing the protein expression of the p85 subunit of PI3-kinase in muscle and adipose tissue and subsequently reducing PI3-kinase signalling [72, 73].

In our study, we observed that GH-V treatment via SC significantly increased fasting plasma insulin concentrations at the 5 mg/kg dose, with no corresponding changes in fasting glucose concentrations. A dose-dependent increase in fasting insulin concentration and IR was also observed in all treatment groups via SC and OP. We investigated the mRNA expression levels of components of insulin signalling pathway in the liver and found that hepatic Irs, Insr, Akt2, Akt3, Pik3ca, Pik3r1, Glut1, Glut2 and Glut4 expression were not significantly different to vehicle after GH-V treatment via either delivery modes, but Akt1 expression in the SC group was significantly increased.

AKT is a downstream target of PI3-kinase signalling and mediates most of the PI3-kinase-mediated metabolic actions, including the translocation of GLUT4. In mammals, AKT has three isoforms, encoded by different genes (Akt1-3). These isoforms have some differential and non-redundant physiological functions, suggested by phenotypes of mice-lacking individual AKT isoforms. Akt1 is essential for cell survival and body growth, but dispensable
for maintenance of glucose homeostasis in mice [74]. In contrast, AKT2 deficient mice display IR and diabetes phenotype, contributed by the inability of insulin to induce glucose utilization and decrease hepatic glucose output [75]. AKT3, however, has no proven metabolic effects.

Previously we tested GH-V in normal pregnant mice and found that hepatic expression of Glut4 gene was significantly down-regulated with concomitant hyperinsulinemia [76]. A trend for decreased Glut4 expression was also seen in the present study. The IR induced by GH-V may result from reduced expression of Glut4, which has been associated with IR [36, 77]. Altered mRNA or protein expression may occur in other insulin target tissues, including muscle [78, 79] and adipose tissue [80, 81], and is currently under investigation.

The secretion of GH from the pituitary is controlled by a number of neuronal, hormonal and metabolic factors. In addition, GH and IGF-1 have a feedback effect, either alone or in combination, to inhibit GH secretion [82]. It is commonly believed that exogenous GH treatment suppresses endogenous GH secretion, although the mechanism is unclear, as are the sites of action. It is, therefore, possible that different delivery modes of exogenous GH-V impacted differently on the secretion of endogenous GH, and may have contributed to different responses between pulsatile and continuous treatments that we observed. In order to clarify this, further studies in GH-deficient models are warranted, to control for the effects from endogenous GH.

This is the first study, to our knowledge, to investigate the effects of different modes of GH-V treatment in mice. In conclusion, pulsatile and continuous administration of GH-V had similar effects on the variables described; pulsatile treatment was more effective in stimulating growth but caused marked hyperinsulinemia. Independently from the mode of GH administration, the time, dose and duration of GH exposure, the sex and strains of animals may all contribute to those variations observed in previous studies. Additional studies are necessary to evaluate the differential effects between GH-V and GH-N.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

References


42. W.R. Baumbuch, B. Bingham, One class of growth hormone (GH) receptor and binding protein messenger ribonucleic acid in rat liver, GHR1, is sexually dimorphic and regulated by GH. Endocrinology 136(2), 749–760 (1995). doi:10.1210/end.136.2.7835307


48. F. Sanchez-Jimenez, P.J. Fisher, R.R. Martinez, W.C. Smith, F. Talamantes, Hypophysectomy eliminates and growth hormone (GH) maintains the midpregnancy elevation in GH receptor and


Appendix III

Human placental growth hormone is increased in maternal serum at 20 weeks of gestation in pregnancies with large-for-gestational-age babies

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Abstract
To investigate the relationship between maternal serum concentrations of placental growth hormone (GH-V), insulin-like growth factor (IGF)-1 and 2, IGF binding proteins (IGFBP)-1 and 3 and birth weight in appropriate-for-gestational-age (AGA), large-for-gestational-age (LGA) and small-for-gestational-age (SGA) cases in a nested case-control study. Maternal serum samples were selected from the Screening for Pregnancy Endpoints (SCOPE) biobank in Auckland, New Zealand. Serum hormone concentrations were determined by ELISA. We found that maternal serum GH-V concentrations at 20 weeks of gestation in LGA pregnancies were significantly higher than in AGA and SGA pregnancies. Maternal GH-V concentrations were positively correlated to birth weights and customized birth weight centiles. Our findings suggest that maternal serum GH-V and IGFBP-1 concentrations at 20 weeks’ gestation are associated with fetal growth.

Introduction
Delivery of infants with an appropriate birth weight for gestational age is a goal of obstetric care. However, inappropriate fetal growth, either large-for-gestational-age (LGA) or small-for-gestational-age (SGA), is common and clinically relevant as they are linked with a number of perinatal complications. LGA is associated with higher rates of Cesarean birth, shoulder dystocia, postpartum hemorrhage and neonatal hypoglycemia (Weissmann-Brenner et al., 2012), while SGA infants are more likely to be stillborn, and develop perinatal asphyxia, hypothermia and abnormal neurologic symptoms (Doctor et al., 2001; Flenady et al., 2011). Furthermore, both LGA and SGA have been reported to increase the risk for developing certain diseases in later life, such as obesity, type 2 diabetes, hypertension and dyslipidemia (Barker et al., 1990; Eriksson et al., 2001; Gluckman & Hanson, 2004; Lithell et al., 1987). The protein sequences of GH-N and GH-V are highly conserved (93% amino acid identity); however, they have distinct expression profiles. GH-N is mainly secreted in a pulsatile fashion from the pituitary, while GH-V is secreted from the placenta tonically during human pregnancy (Eriksson et al., 1989). The pituitary protein, GH-N (22 kDa), is the primary form of GH in maternal circulation up to 15–20 weeks of gestation (Als et al., 1997). During human pregnancy, GH-V is detected in the maternal circulation from as early as week 5, gradually replacing maternally derived GH-N as the dominant circulating form of GH at approximately 20 weeks of gestation (Als et al., 1997; Eriksson et al., 1989). After that, GH-V concentrations increase significantly to reach a peak at approximately 37 weeks of gestation (Chellakooty et al., 2004; Franklin et al., 1988). Previous studies have demonstrated a positive relationship between maternal GH-V serum concentration and fetal growth (Chellakooty et al., 2004; Handwerger & Freemark, 2000; McIntyre et al., 2000; Mirlesse et al., 1993; Mittal et al., 2007; Pedersen et al., 2010; Sifakis et al., 2012). Consistently, decreased concentrations of circulating GH-V, and reduced placental mRNA expression of this hormone, have been observed in mid to late stage growth restricted pregnancies, compared to normal pregnancies (Koutsaki et al., 2011; McIntyre et al., 2000). However, whether there is an association between maternal concentration...
of GH-V earlier in pregnancy and infant birth weight at term is not clear. In the present study, we therefore aimed to examine the relationship between maternal serum GH-V concentrations at 20 weeks of gestation and infant birth weight. We hypothesized that maternal serum GH-V concentrations were altered in pregnancies with inappropriate growth babies. Maternal serum insulin-like growth factor (IGF)-1, IGF-2, IGF binding proteins (IGFBP)-1 and IGFBP-3 concentrations were also measured to investigate potential relationships between GH-V and other primary components of the IGF-IGFBP system.

Materials and methods

Ethical approval was obtained from New Zealand Health and Disability Ethics Committees (AKX/02/00/364/AM03), and all women provided written informed consent. Between November 2004 and October 2007, 2032 nulliparous women with singleton pregnancies were recruited to the Screening for Pregnancy Endpoints (SCOPE) study in Auckland, New Zealand. The inclusion criteria have been described previously (McCowan et al., 2007).

Participants were interviewed and examined by a SCOPE research midwife at 15 and 20 weeks of gestation. At the first visit, detailed clinical and demographic data were collected and entered into an internet accessed, central database with a complete audit trail (MedSciNet, Stockholm, Sweden). Umbilical artery resistance index (RI) and mean uterine artery RI were measured using Doppler ultrasound at 20 weeks. Maternal serum samples were collected at 20 weeks and stored at −80°C for subsequent analyses. Birth weight was recorded using electronic scales at the time of birth.

In this nested case-control study, 50 LGA and 49 SGA cases were selected and matched to 50 controls (appropriate-for-gestational-age, AGA), matched by ethnicity. No women included in this study had pregnancies complicated by gestational diabetes or gestational hypertensive disease. Customized birth weight centile was calculated, adjusted for mother’s height and weight at 15 weeks’ visit, ethnicity, sex and gestation at delivery. SGA and LGA were defined as birth weight <5th and >95th customized birth weight centiles, respectively (McCowan et al., 2004).

Materials

Recombinant human GH-V (22 kDa) was purchased from Protein Laboratories Rehovot (Rehovot, Israel) and was reconstituted in 0.4% NaHCO3 pH 9 (Solomon et al., 2006). Recombinant human GH-N (22 kDa) and placental lactogen (PL) were obtained from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA). Human GH-V monoclonal antibodies E8 (MCA5827G) and 7C12 (MCA5828G) were obtained from Bio-Rad AbD Serotec (Raleigh, NC). E8 does not cross react with GH-N or prolactin. 7C12 shows some cross reactivity with GH-N (5%) as per details provided by the manufacturer.

The development of GH-V ELISA

Due to the lack of a sensitive and specific commercially available assay, an in-house sandwich ELISA was developed and validated for the measurement of GH-V in serum. Mouse anti human GH-V antibodies E8 and 7C12 were tested for specificity for GH-N, GH-V and PL using an indirect ELISA (Supplementary Figure 1). E8 was used as the capture antibody due to its binding specificity for GH-V (Evain-Brion et al., 1994; Igout et al., 1993). 7C12 was used as the detection antibody, and biotinylated using a LYNX Rapid Biotin (Type 1) Antibody Conjugation Kit (Bio-Rad AbD Serotec) according to the manufacturer’s instructions. Optimal capture and detection antibody concentrations were determined through standard checkerboard titration procedure (Crowther, 1995). A checkerboard titration experiment was conducted empirically using starting concentrations of 8 and 16 µg/ml for capture and detection antibodies respectively, with the antigen (GH-V) at a constant concentration of 5 ng/ml. The optimal capture and detection antibody concentrations were 2 and 8 µg/ml, respectively. Horseradish peroxidase conjugated streptavidin (Bio-Rad AbD Serotec) was used at a concentration of 200 ng/ml as no significant differences were seen at higher or lower concentrations. Two percent and 5% bovine serum albumin (BSA), 2% and 5% nonfat dry milk, and a commercial blocking buffer (Ultrablock, Bio-Rad AbD Serotec) were tested under identical ELISA conditions. Ultrablock was found to have the lowest signal-to-noise ratio and was used as the blocking buffer and sample diluent in subsequent experiments. Serum samples from a healthy donor, and sample diluent, were spiked with GH-V at a concentration of 0.625 ng/ml and serial dilutions (neat, 1:2, 1:4 and 1:8) from unspiked, spiked and control samples were assayed. Recovery rate was calculated as the following formula: % Recovery = (% Spiked sample values – Unspiked sample values)/Expected values × 100. The average recovery rate of this assay was 106%. Linearity was determined by calculating the recovery rate of neat, 1:2, 1:4 and 1:8 dilutions in spiked and unspiked samples, and fell within the acceptance range of 80–120%. Parallelism between the GH-V standards and serial dilutions of serum samples indicated that the standard accurately reflects the GH-V content in natural samples (Supplementary Figure 2). Coefficients of variation (CV) of intra-assay and inter-assay were 4.8% and 6.8%, respectively.

The GH-V ELISA procedure

Microtiter plates were coated with antibody E8 diluted in phosphate buffer (0.1 M Sodium Carbonate, pH 9.5) at a concentration of 2 µg/ml by overnight incubation at 4°C. Coated plates were washed three times with wash buffer (PBS-T; 10 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.05% Tween 20). Blocking was achieved by 1 h incubation at room temperature with Ultrablock. Standards were prepared from GH-V solution with a range from 0.078 to 5.0 ng/ml. Standards and neat serum samples were incubated for 2 h at room temperature, then washed three times. All serum samples were measured in duplicate. Biotinylated 7C12 (8 µg/ml) was added and incubated for 1 h. The plates were washed three times and 200 ng/ml horseradish peroxidase conjugated streptavidin was added and incubated for 30 min. The microtiter plates were then washed four times and 3, 3′, 5, 5′-tetramethylbenzidine (TMB) Substrate Reagent Set (BD
Biosciences, San Jose, CA) was added to the wells producing a visible signal that is correlated with the amount of antigen. Absorbance was read at 450 nm and 590 nm within 30 min of stopping the reaction.

Serum analysis
Serum IGF-1, IGF-2, IGFBP-1 and IGFBP-3 were assayed with human-specific enzyme-linked immunosorbent assays (ELISA) as per the manufacturer’s instructions (Mediagnost, Reutlingen, Germany).

Statistical analysis
Concentrations of GH-V, IGF-2, IGFBP-1 and IGFBP-3 were positively skewed. Outliers were defined as data points more than 1.5× the interquartile range above the upper or below the lower quartile. Data were log-transformed to improve the approximation of normal distribution and linearize relationships. Data are expressed as means± S.E.M. and median unless stated otherwise. Group means were compared using one-way ANOVA with post hoc analysis (Tukey’s procedure). Categorical and discrete numerical variables were compared using chi-square or Fisher’s exact test. Pearson’s coefficient was used to determine correlations between variables. Multiple regression was used to determine the association of maternal hormone concentrations with birth weight after controlling for known clinical correlates. All analyses were conducted using IBM SPSS Statistics 21 (IBM Corp., Armonk, NY). A p value of <0.05 was accepted as statistically significant.

Results
The demographic and clinical details of the three groups are shown in Table 1. SGA babies were born earlier with smaller placenta. The mean uterine artery RI at 20 weeks was significantly higher in women destined to deliver SGA placentas. The mean uterine artery RI at 20 weeks was shown in Table 1. SGA babies were born earlier with smaller The demographic and clinical details of the three groups are

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Results expressed as mean (SEM) if not indicated; ns: not significant; BMI: body mass index.

Concentrations of maternal hormones
Maternal serum GH-V concentrations varied between individuals. A few samples had absorbance values falling out of the range of the standard curve. The concentration of these samples was extrapolated from the standard curve, as insufficient samples were available for repeat measurements. Subsequently those extrapolated data were identified as outliers and were not included in the group comparisons (Figure 1A,B). Maternal serum GH-V concentrations at 20 weeks of gestation were significantly higher in LGA pregnancies (median concentration, 2.07 ng/ml) compared to AGA (1.72 ng/ml) (p < 0.05) or SGA pregnancies (1.65 ng/ml) (p < 0.05), however, there was no significant difference between SGA and AGA samples (Figure 1A,B). There were no significant differences in IGF-1, IGF-2, IGFBP1 and IGFBP-3 concentrations between the groups (Figure 1C–F). Infant gender did not affect the maternal concentrations of GH-V, IGFs and IGFBPs at 20 weeks (data not shown).

Correlation analysis
One of the aims of this study was to determine whether estimations of maternal GH-related parameters were related to birth weights (Table 2). In the correlation analysis, there was a weak but significant positive relationship of maternal GH-V concentrations with birth weights (r = 0.176, p = 0.033) (Figure 2A), birth weights adjusted for gestational age (r = 0.174, p = 0.035) and customized birth weight centiles (r = 0.163, p = 0.046) (Table 2), as well as placental weights (r = 0.233, p = 0.011). The mean uterine artery RI at 20 weeks was negatively associated with birth weight (r = −0.414, p < 0.0001), birth weight adjusted for gestational age (r = −0.402, p < 0.0001) and customized birth weight centile (r = −0.38, p < 0.0001). Maternal IGF-1 concentrations were related to the changes in GH-V (r = 0.343, p < 0.0001) (Figure 2B), and weakly correlated to changes in IGF-2 (r = −0.168, p = 0.042) and the changes in IGFBP-3 (r = 0.187, p = 0.027). There were no correlations between maternal IGF-I, IGF-2, IGFBP-3 and birth weights. However, maternal IGFBP-1 showed a weak positive relationship with mean uterine artery RI (r = 0.258, p = 0.002) and a weak
inverse relationship with birth weights ($r = -0.257$, $p = 0.002$) (Figure 2C), birth weights adjusted for gestational age ($r = -0.253$, $p = 0.002$) and customized birth weight centiles ($r = -0.210$, $p = 0.011$) (Table 2).

Multiple regression was used to determine whether any GH related variables were associated with birth weight after controlling for maternal age, ethnicity, socioeconomic status, smoking and drinking habits, maternal BMI, infant sex and gestation at delivery. Maternal GH-V and IGFBP-1 concentrations were significantly associated with birth weights in separate models that controlled for those factors. Using GH-V and IGFBP-1 in combination with each other, or in combination with the IGF-1, IGF-2 or IGFBP-3 did not improve the model.

Discussion

In the present study, we examined maternal GH-V, IGF-1, IGF-2, IGFBP-1 and IGFBP-3 at 20 weeks of gestation in the maternal samples in pregnancies that later resulted in AGA, SGA or LGA births. This approach has allowed determination of the correlation between GH-related hormones and their relationships with birth weight. We found that maternal serum GH-V was increased at 20 weeks in LGA pregnancies, and that GH-V was positively associated with birth weight.

Most of the studies in mid-late pregnancy found a positive association between maternal GH-V and fetal growth. Chellakooty et al. observed that the change in GH-V at 24.5–37.5 weeks was positively associated with fetal growth rate and birth weight (Chellakooty et al., 2004). Two studies reported lower GH-V concentrations at approximately 30 weeks in pregnancies complicated by fetal growth restriction (McIntyre et al., 2000; Mirlesse et al., 1993). Women with...
Infants in the AGA range are presumably healthy. Further, it cannot distinguish one process from the other, and those definition of LGA and SGA is following a pathological process or may represent constitutionally big or small infants. The definitions of LGA and SGA can either occur as early as 20 weeks, has a positive relationship with birth weight. However, both LGA and SGA can usually be assessed by sonographic measurements (Pedersen et al., 2010). However, this study found that maternal GH-V at that period was not associated with birth weight in either SGA or normal pregnancies (Sifakis et al., 2012). Studies that showed no relationship may have been limited by issues of sample size and/or the time of sampling. Our study provides further evidence that maternal GH-V concentration at mid-gestation. Limitations of studies have investigated associations of circulating form of GH at approximately 20 weeks of gestation. Limited studies have investigated associations of GH-V with fetal growth at earlier time points. Pedersen et al. observed increased GH-V concentration in women at weeks 11–14 carrying fetuses with high growth rates assessed by sonographic measurements (Pedersen et al., 2010). However, another study found that maternal GH-V at that period was not associated with birth weight in either SGA or normal pregnancies (Sifakis et al., 2012). Studies that showed no relationship may have been limited by issues of sample size and/or the time of sampling. Our study provides further evidence that maternal GH-V concentration at mid-gestation, as early as 20 weeks, has a positive relationship with birth weight. However, both LGA and SGA can usually occur following a pathological process or may represent constitutionally big or small infants. The definitions of LGA and SGA cannot distinguish one process from the other, and those infants in the AGA range are presumably healthy. Further, GH-V has been found to be associated with a number of pathological conditions, such as pre-eclampsia (Mittal et al., 2007), gestational diabetes (McIntyre et al., 2000) and Down’s syndrome (Baviera et al., 2004). In this study, we found an association of GH-V with birth weight in LGA, AGA and SGA cases. Whether this association reflects the “normal” or pathological changes of birth weight is still unclear.

GH-V is thought to play a key role in maternal adaptation to pregnancy and fetal growth (Newbern & Freemark, 2011). First, GH-V promotes the adaptation to pregnancy of blood vessels supplying the placenta (Lacroix et al., 2005), and relaxes the arteries supplying the uterus (Schiessl et al., 2007); the effect of these changes is an increase in blood flow to the fetus. Second, GH-V shares similar physiological somatotropic, lactogenic and lipolytic properties with GH-N (Alsat et al., 1997; Verhaeghe, 2008). The growth-promoting effect of GH-V has also been demonstrated in vivo in non-pregnant hypophysectomized rats treated with GH-V and transgenic mice (Barbour et al., 2002; MacLeod et al., 1991; Selden et al., 1988). Third, GH-V increases maternal concentrations of other important growth factors, such as IGF-1 (Caufriez et al., 1990, 1994). A highly significant correlation between GH-V and IGF-1 was also found in the present study. Moreover, GH-V has been proposed to be a likely candidate to mediate the insulin resistance of pregnancy in transgenic mice (Barbour et al., 2002), where GH-V may impact on maternal metabolism and substrate supply to the fetus, either directly or mediated by IGF-1.

IGF-1 and IGF-2 mediate a range of actions in many tissues including stimulation of cell growth, cell survival and differentiation. These actions are regulated by a series of specific binding proteins, which may inhibit or enhance IGF activity (Baxter, 2000). There is clear evidence that the IGFs and IGFBP family are closely related to fetal growth (Boyne et al., 2003; Chard, 1994; Han et al., 1996; Hills et al., 1996; Holmes et al., 1997), however, their associations were mostly determined at mid to late pregnancy. In the present study, we found that maternal serum IGFBP-1 as early as 20 weeks was negatively related to birth weight, but IGF-1, IGF-2 and IGFBP-3 had no association with birth weight at that time. Maternal serum IGFBP-1 may therefore be a potential biochemical marker for early detection of inappropriate fetal growth.

IGFBP-1 binds to only a small proportion of circulating IGF-1 (Frystyk et al., 2002). However, it is considered to be important for short-term regulation of IGF bioactivity, since IGFBP-1 concentrations fluctuate in response to insulin and carbohydrate intake (Baxter, 1995). Previous studies have demonstrated two major roles of IGFBP-1. IGFBP-1 serves as an endocrine factor to regulate the bioavailability of serum IGF-1, inhibits IGF binding to cell surface receptors, and thereby inhibits IGF-mediated cell mitogenic and metabolic actions. Such a mechanism can be reconciled with observations showing that serum concentrations of “free” IGF-1 are markedly increased during late pregnancy, consequent on IGFBP-3 proteolysis and decreased ternary complex formation (Skjaerbaek et al., 2004). The IGF inhibitory actions of IGFBP-1 have been confirmed by in vitro studies and in vivo animal investigations (Jones et al., 1991; Lee et al., 1993; 1992).
Lowman et al., 1998; Rajkumar et al., 1995). Overexpression and secretion levels of circulating IGFBP-1 result in inhibition of fetal growth and the metabolic effects of the IGFs (Murphy et al., 1995; Rajkumar et al., 1995), while low IGFBP-1 concentrations are associated with macrosomia and insulin resistance syndromes (Heald et al., 2001; Janssen et al., 1998; Yan-Jun et al., 1996). Consistent with previous studies (Harrington et al., 1997; Melchiorre et al., 2009), we found that uterine artery RI at 20 weeks was increased in pregnancies with SGA and was negatively associated with birth weight. In addition, a positive relationship between IGFBP-1 and uterine artery RI was observed. A recent study revealed that IGFBP-1 induced vasodilatation independently of IGF by increasing endothelial nitric oxide synthase activity in mice (Rajwani et al., 2012). However, the effect of IGFBP-1 on the uteroplacental vessel tone during pregnancy is still unclear. The other role of IGFBP-1 is to act as an autocrine/paracrine factor in the female reproductive system (Rutanen & Seppala, 1992). In humans, IGFBP-1 is synthesized in large amounts by the secretory endometrium, ovarian granulosa cells and decidualized stromal endometrial cells of early pregnancy (Koistinen et al., 1990; Rutanen et al., 1985). IGFBP-1, together with IGFs, ovarian steroids and other factors, are involved in a complex system which regulates decidualization, trophoblast invasion, and fetal growth (Hamilton et al., 1998; Han et al., 1996).

In conclusion, maternal serum GH-V and IGFBP-1 concentrations at 20 weeks of gestation were significantly associated with birth weight. However, given the strength of the association, a potential clinical use as early markers for aberrant fetal growth patterns would require further investigation. As an association between maternal circulating GH-V and SGA has been observed in the third trimester, measurements taken later in pregnancy may be more informative.

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Declaration of interest
The authors have nothing to declare.

References
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Supplementary material available online.