{Energy Homeostasis in children with Prader-Willi syndrome}
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{MBBS, PGDTMH, FRACP}

A thesis submitted for the degree of Doctor of Philosophy at
The University of Queensland in {2015}
{School of Medicine}
Abstract

Introduction
Prader-Willi syndrome (PWS) is a genetic condition commonly associated with hyperphagia and obesity. PWS is thought to have hypothalamic dysfunction which is the head ganglion of autonomic nervous system (ANS). In current literature, ANS is believed to be defective in PWS. ANS may also have a role in controlling orexigenic hormone ghrelin and energy expenditure. One study reported higher resting energy expenditure adjusted for lean body mass in growth hormone naive PWS group but another study found lower activity associated energy expenditure compared to controls. Other studies found adjusted basal and sleeping metabolic rates were not different to the controls. Complete profile of energy expenditure in PWS remains unclear.
We hypothesize that there is defective ANS in PWS, as a result of hypothalamus dysfunction, and it leads to high orexigenic hormone, acyl ghrelin, and low energy expenditure that in turn cause obesity.

Methods
We compared the ANS functions, acyl ghrelin status and energy expenditure in children with PWS and controls.
We recruited 16 genetically-confirmed children with PWS and 16 controls. Exclusion criteria were diabetes mellitus, psycho-trophic medications, and other hypothalamic pathologies.
We performed a mixed meal challenge to assess ANS function and acyl ghrelin status of PWS and control groups. We used Bodystat 1500® to measure body composition. Orthostatic hypotension, due to gravity, stimulates baroreceptors and activates sympathetic nervous system to counter regulate postural drop in blood pressure by increasing pulse rate, stroke volume and vasoconstriction. We used orthostatic change in pulse rate (PR), blood pressure (BP), and mean arterial pressure (MAP) expressed as per cent change of PR (%ΔPR), BP (%ΔBP), and MAP (%ΔMAP) from lying to standing to access sympathetic nervous function. ANS was further stimulated by a mixed meal and we examined %ΔPR, %ΔBP, and %ΔMAP at 15 and 30 seconds after standing from recumbent position; at fasting, and post-prandial periods. We also measured plasma gastrin, catecholamines (Pcat) and urinary catecholamines (Ucat) at fasting and post-prandial periods to complement autonomic cardiovascular data. Using Actiheart®, we compared weight and fat free mass adjusted total, resting, activity-associated and non-exercise associated thermogenesis between two groups.
Results

PWS group was younger, shorter, and had reduced lean mass than the controls. Post-prandial %ΔPR at both 15 and 30 seconds were significantly lower in PWS group than controls. The difference in %Δ systolic BP and diastolic BP did not reach statistical significance but %ΔMAP at 60 min and 120 min after meal was significantly lower in PWS. Postprandial plasma gastrin and Ucat were higher in PWS group than controls but Pcat were not different in two groups. Fasting plasma acyl ghrelin (AG) was significantly higher in PWS but it decreased to similar level of controls at 60 and 120 minutes after a meal. The rate of fall of plasma acyl ghrelin was faster in the PWS group than the controls. Fasting AG is negatively correlated to fasting %ΔPR at 30s (r value -0.52, p= 0.04).

When adjusted for both weight and fat-free mass, PWS group had lower total, resting, activity-associated and non-exercise associated thermogenesis than the controls.

Conclusions

We report that there is dysautonomia, high fasting acyl ghrelin and low energy expenditures in children with PWS.

In PWS, there is reduction in GABA-A receptor number and its actions as a result of the deleted genes of β3, α5, and γ3 subunit of GABA-A receptors in the PWS gene region; and probable exaggerated GABA-B receptors actions due to effect of compensatory hyper-γ-amino-butyric-acidaemia on the normal GABA-B receptors. The abnormities lead to GABA system dysfunction in PWS. GABA is the key neurotransmitter between Nucleus Tractus Solitarius and C1 neurons that connect to the thoracic spinal cord that sends efferent neurons to sympathetic ganglions. GABA system dysfunction, therefore, may be the cause of sympathetic failure. Moreover, GABA is generally an inhibitory neurotransmitter and GABA dysfunction may be the cause of poor vagal inhibitory function that lead to high post-prandial plasma gastrin production, and increased catecholamine production from adrenal medulla probably by increase chromaffin cells gap junction communications. Our findings of dysautonomia can be explained by GABA dysfunction in PWS. Dysautonomia may also be the cause of high fasting acyl ghrelin and low energy expenditures. Therefore in PWS, there is imbalance in energy intake and expenditure resulting in obesity.
Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

1. Benefit of early commencement of growth hormone in children with Prader-Willi Syndrome
   **Ohn Nyunt**, Ian Hughes, Mark Harris, Tony huynh, Peter SW Davies, Andrew M Cotterill
   (J Pediatr Endocrinol Metab 22(12):2009: 1151-8.)

2. Normal low dose Synacthen test in children with Prader-Willi Syndrome

3. Central sleep-disordered breathing and the effects of oxygen Therapy in infants with Prader-Willi syndrome

Conference presentations

Presentations in 2009

1. “Bone age advancement in children with Prader-Willi syndrome”
   **O Nyunt**, Patricia H Galligo, SM Archbold, JM Donnelly, AM Cotterill, M Harris
   Poster presentation at Lawson Wilkins Pediatric Endocrine Society/ European Society for Paediatric Endocrinology 8th Joint Meeting at New York, USA

2. “Case reports of premature adrenarche in children with Prader-Willi syndrome”
   **Ohn Nyunt**, Sinead M Archbold, Jennifer M Donnelly, Andrew M Cotterill, Mark Harris
   Poster presentation at Lawson Wilkins Pediatric Endocrine Society/ European Society for Paediatric Endocrinology 8th Joint Meeting at New York, USA

3. “BMI changes and metabolic factors in children with Prader-Willi syndrome”
   **Ohn Nyunt**, Sinead M Archbold, Jennifer M Donnelly, Andrew M Cotterill, Mark Harris, Gary M Leong
   Poster presentation at Lawson Wilkins Pediatric Endocrine Society/ European Society for Paediatric Endocrinology 8th Joint Meeting at New York, USA
4. “Assessment of adrenocorticotropic deficiency in children with Prader-Willi syndrome”

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Poster presentation at Lawson Wilkins Pediatric Endocrine Society/ European Society for Paediatric Endocrinology 8th Joint Meeting at New York, USA

**Presentations in 2010**

Authors: Jennifer Donnelly, Sinead Archbold, **Ohn Nyunt**
Poster presentation at the International Prader-Willi syndrome Organisation 7th Scientific Conference, Taipei, ROC

2. “Orthopaedic Manifestations of children with Prader-Willi syndrome”
Authors: **O. Nyunt**, A. Gupta, M. Harris, J. Walsh, A.M. Cotterill
Poster presentation at the International Prader-Willi syndrome Organisation 7th Scientific Conference, Taipei, ROC

**Presentation in 2012**

“Appetite hormones and energy expenditure in children with Prader-Willi syndrome”

*(Presentation of the research project)*

**Authors: O Nyunt**, SM Archbold, JM Donnelly, D Longmore, PL Jefferies, GM Leong, AM Cotterill, M Harris and PS Davies
Oral presentation at the second Asia-Pacific Prader-Willi syndrome Conference, Sydney, Australia

**Presentation in 2014**

“Dysautonomia and acyl ghrelin in Prader-Willi syndrome”

*(Presentation of the thesis)*

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Poster presentation at European Society of Paediatric Endocrinology (ESPE) 2014, Dublin, Ireland
Presentations in 2015

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*(Presentation of the thesis)*

**Authors:** O Nyunt, SM Archbold, JM Donnelly, P Jeffery, AM Cotterill, M Harris and PS Davies

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**Publications included in this thesis**

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Contributions by others to the thesis
Conception of the project: Dr. Mark Harris and A/ Prof. Andrew Cotterill
Research methods: Dr. Mark Harris, A/ Prof. Andrew Cotterill, Dr. David Cowley and Prof. Frank Bowling
PWS research team: Mrs. Sinead Archbold and Mrs. Jenny Donelly
Laboratory work for acyl ghrelin: Dr. Penny Jeffaries
Statistics: Prof. Peter Davies

Statement of parts of the thesis submitted to qualify for the award of another degree
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prader-willi syndrome, autonomic nervous system, acyl ghrelin, energy expenditure, gamma amino butyric acid, gastrin, catecholamine

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ANZSRC code: 110306, Clinical Sciences: Endocrinology, 50%
ANZSRC code: 111403, Paediatrics and Reproduction: Paediatrics, 20%

Fields of Research (FoR) Classification
FoR code: 1101, Medical Biochemistry and Metabolomics, 30%
FoR code: 1103, Clinical Sciences, 50%
FoR code: 1114, Paediatrics and Reproduction, 20%
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<th>Description</th>
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<tr>
<td>ΔDBP or DDBP</td>
<td>change in diastolic blood pressure</td>
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<tr>
<td>ΔMAP</td>
<td>change in mean arterial pressure</td>
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<tr>
<td>ΔPR or DPR</td>
<td>change in pulse rate</td>
</tr>
<tr>
<td>ΔSBP or DSBP</td>
<td>change in systolic blood pressure</td>
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<td>AAT</td>
<td>activity associated thermogenesis</td>
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<tr>
<td>Acad1</td>
<td>acetyl coA dehydrogenase long chain</td>
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<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
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<td>Agrp</td>
<td>Agouti-related peptide</td>
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<td>AHI</td>
<td>apnoea- hypopnea index</td>
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<td>Alas1</td>
<td>aminolevulinic acid synthase 1</td>
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<tr>
<td>ANS</td>
<td>autonomic nervous system</td>
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<tr>
<td>APEG</td>
<td>Australasian Paediatric Endocrine Group</td>
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<td>ARC</td>
<td>Arcuate nucleus</td>
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<td>AS</td>
<td>Angelman syndrome</td>
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<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>AUC</td>
<td>area under curve</td>
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<tr>
<td>BAT</td>
<td>brown adipose tissue</td>
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<td>Bdh</td>
<td>3 hydroxy butyrate dehydrogenase</td>
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<td>BMR</td>
<td>basal metabolic rate</td>
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<td>BNDF</td>
<td>brain-derived neurotropic factor</td>
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<tr>
<td>BP&lt;sub&gt;1,2,3&lt;/sub&gt;</td>
<td>break points 1,2, and 3</td>
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<td>CART</td>
<td>cocaine and amphetamine- regulated transcript</td>
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<td>CI</td>
<td>central index</td>
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<td>CoA</td>
<td>coenzyme A</td>
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<tr>
<td>CoQ10</td>
<td>coenzyme Q10</td>
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<td>DMV</td>
<td>dorsal motor nucleus of Vagus</td>
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<td>EAT</td>
<td>exercise associated thermogenesis</td>
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<td>EE</td>
<td>energy expenditure</td>
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<td>FADH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>flavin adenine dinucleotide</td>
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<td>FISH</td>
<td>fluorescence in-situ hybridization</td>
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<td>FMZ</td>
<td>Flumazenil</td>
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<td>GABA</td>
<td>Gamma amino- butyric acid</td>
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<td>GH</td>
<td>growth hormone</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>GHT</td>
<td>Growth hormone therapy</td>
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<td>HERC2</td>
<td>HECT domain and RCC1-like domain 2</td>
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<td>HPA</td>
<td>Hypothalamo-pituitary-adrenal</td>
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<tr>
<td>IC</td>
<td>Imprinting center</td>
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<tr>
<td>IGF1</td>
<td>Insulin like growth factor 1</td>
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<td>IPW</td>
<td>Imprinted in PWS</td>
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<td>IX</td>
<td>Glossopharyngeal nerve</td>
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<td>IncRNA</td>
<td>Long non-coding RNA</td>
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<td>MAGEL-2</td>
<td>Mage-like 2</td>
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<td>MAP</td>
<td>Mean arterial pressure</td>
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<td>MC4R</td>
<td>Melanocortin 4 receptor</td>
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<tr>
<td>MCH</td>
<td>Melanin-concentrating hormone</td>
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<td>MKRN3</td>
<td>Makorin-3</td>
</tr>
<tr>
<td>MKRN3-AS</td>
<td>Makorin-3- Angelman syndrome</td>
</tr>
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<td>Mrpl15</td>
<td>Mitochondrial ribosomal protein L15</td>
</tr>
<tr>
<td>MSH</td>
<td>Melanocyte stimulating hormone</td>
</tr>
<tr>
<td>MS-MLPA</td>
<td>Methylation specific multiplex ligation-dependent probe amplification</td>
</tr>
<tr>
<td>MTCYB</td>
<td>Cytochrome b of complex III</td>
</tr>
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<td>Mte1</td>
<td>Mitochondrial acetyl CoA thioesterase 1</td>
</tr>
<tr>
<td>MTOR</td>
<td>Mechanistic target of Rapamycin</td>
</tr>
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<td>NADH</td>
<td>Reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NCD</td>
<td>Necdin</td>
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<tr>
<td>NEAT</td>
<td>Non-exercise associated thermogenesis</td>
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<td>NPY</td>
<td>Neuropeptide Y</td>
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<td>NTS</td>
<td>Nucleus tractus solitarius</td>
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<td>Oculo-cutaneous albinism 2</td>
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<td>Obstructive index</td>
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<td>POMC</td>
<td>Pro-opiomelanocortin C</td>
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<td>PSNS</td>
<td>Parasympathetic nervous system</td>
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<tr>
<td>PVN</td>
<td>Paraventricular nucleus of hypothalamus</td>
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<td>PWS</td>
<td>Prader-Willi syndrome</td>
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<td>PWS-IC</td>
<td>PWS imprinting center mutant</td>
</tr>
<tr>
<td>REE</td>
<td>Resting energy expenditure</td>
</tr>
<tr>
<td><strong>RNA</strong></td>
<td>ribonucleic acid</td>
</tr>
<tr>
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<tr>
<td><strong>Snord</strong></td>
<td>small nucleolar RNA, C/D box</td>
</tr>
<tr>
<td><strong>SnoRNA</strong></td>
<td>small nucleolar ribonucleic acid</td>
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<tr>
<td><strong>SNS</strong></td>
<td>sympathetic nervous system</td>
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<tr>
<td><strong>SNURF-SNRPN</strong></td>
<td>small nuclear ribonucleoprotein N</td>
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<tr>
<td><strong>TEE</strong></td>
<td>total energy expenditure</td>
</tr>
<tr>
<td><strong>THR</strong></td>
<td>thyroid hormone receptor</td>
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<tr>
<td><strong>UB3A-AS</strong></td>
<td>ubiquitin- protein ligase E3A</td>
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<tr>
<td><strong>UCP</strong></td>
<td>uncoupling protein</td>
</tr>
<tr>
<td><strong>X</strong></td>
<td>Vagus nerve</td>
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Chapter 1: Introduction and hypothesis

1.1: Prader-Willi Syndrome (PWS)

Prader-Willi Syndrome (PWS) is a chromosomal disorder and it is one of the common genetic causes of obesity (Prader, Labhart et al. 1956, Cassidy and Driscoll 2009). It is due to the result of deletion of paternal chromosome 15q11.13 in 75% of PWS (Butler 1990, Bittel and Butler 2005). Typical deletions in PWS occur at either breakpoint 1 (BP1; located between 18.68 and 20.22Mb) or breakpoint 2 (BP2; located between 20.81 and 21.36 Mb) at the centromeric end with the resulting genetic subtypes designated as type I or type II deletions, respectively. Regardless of whether the deletion begins at BP1 or BP2, typical deletions generally end at breakpoint 3 (BP3, located between 25.94 and 27.28 Mb) at the telomeric end of the 15q11-q13 region (Butler, Fischer et al. 2008) (Figure 1).

Figure 1: Genetic map of human chromosome 15q11-13 adapted from (Cassidy and Driscoll 2009, Rout, Abdul-Rahman et al. 2012). Type I deletion is from BP1 to BP3 and type II deletion from BP2 to BP3. Both type I and II deletions in PWS involve GABA-A receptor subunit genes. Gene number 1 to 12 represents MKRN3, MKRN3-AS, MAGEL2, NCD, C150RFN, SNURF-SNRPN, IPW, UB3A-AS, UBE3A, ATP10A, OCA2, and HERC2 respectively. UBE3A and ATP10A are maternally expressed and linked with Angelman syndrome whereas the rest of the genes are paternally expressed and linked with PWS. Imprinting center (IC) is both maternally and paternally expressed. Small nucleolar RNA (snoRNA) contains non-coding RNA such as snord115 and116.

Normally the maternal copy of chromosome 15 is inactivated and the paternal copy is expressed. PWS results when active paternal copy is absent (Nicholls, Knoll et al. 1989). Twenty percent of PWS is due to maternal uniparental disomy and 5% due to imprinting center defect at paternal chromosome 15q11 (Robinson, Bottani et al. 1991, Bittel and Butler 2005) (Figure 2). Maternal uniparental- disomy is thought to occur due to an earlier
embryonic trisomy 15 (two copies of maternal chromosomes 15 and a copy of paternal chromosome 15) followed by trisomy rescue leaving only two copies of maternal chromosome 15. Microdeletion in the imprinting center, which controls the imprinting process within 15q11.13, also leads to development of PWS (Bittel and Butler 2005). The genetic diagnosis for PWS is made by methylation tests, fluorescence in-situ hybridization test (Cassidy and Driscoll 2009), or, more recently, by methylation- specific multiplex ligation- dependent probe amplification (Henkhaus, Kim et al.). Methylation test identifies if the paternal copy of 15q11 is inactivated, and fluorescence in-situ hybridization test examines presence or absence of paternal copy of 15q11 or presence of two maternal copies of 15q11. Methylation- specific multiplex ligation- dependent probe amplification (MS- MLPA- PWS/AS kit from MRC- Holland, Amsterdam, Netherlands) has original kit A1 (25 probes) that is used to detect methylation status and deletion subtypes in individuals with PWS. The newer kit B1 (32 probes) is used for additional information such as imprinting center mutation and adjoining small non- coding RNAs microdeletions (Henkhaus, Kim et al.).

Within the PWS region, there are twelve genes involved, namely small nuclear ribonucleoprotein N (SNURF-SNRPN), makorin-3 (MKRN3 or ZNF127), necdin (NCD) and mage-like 2 (MAGEL-2), MKRN3-AS, C150RFN, imprinted in PWS (IPW), ubiquitin- protein ligase E3A (UB3A-AS), oculo- cutaneous albinism 2 (OCA2), and HECT domain and RCC1- like domain 2 (HERC2) (Cassidy and Driscoll 2009). The genes encode proteins if paternal allele is expressed (Runte, Huttenhofer et al. 2001). A recent work on Snord116del mice showed the long non-coding RNA (lncRNA) in PWS region has regulatory function on gene encoding diurnal rhythm and metabolism (Zhang, Bouma et al. 2012, Powell, Coulson et al. 2013). PWS may be the first human disease that non-coding gene has a role in pathogenesis.

The incidence of PWS by genetic diagnosis in Australia was 1: 25,000 live birth in a study done between 1998 to 2000 (Smith, Egan et al. 2003). In Anglia and Oxford health regions of United Kingdom, the incidence rate was 1: 29,000 live births and the prevalence was 1: 52,000 (Whittington, Holland et al. 2001). In Belgium, the incidence was reported as 1: 26,676 live births and the prevalence 1: 76,574 (Vogels, Van Den Ende et al. 2004).

The common clinical features of PWS include diminished foetal movement during pregnancy, hypotonia, initial poor feeding and failure to thrive, poor growth, developmental delay, hypogonadotrophic hypogonadism, central hypothyroidism, hypopigmentation, small hands and feet, scoliosis, hip dysplasia behaviour problems, sleep disordered breathing, skin picking, dental problems, strabismus, hyperphagia and obesity (Prader,
Classical facial features in PWS include narrow bi-frontal diameter, almond-shape eyes, narrow nasal bridge, and thin upper lip (Prader, Labhart et al. 1956, Cassidy and Driscoll 2009).

During pregnancy mothers with previous normal pregnancies may notice delayed onset and intensity of foetal movements during pregnancy with a foetus that has PWS. It may raise the concern of the foetus having PWS and choriovillous sampling can be used for the genetic diagnosis of PWS.

**Figure 2: Human PWS gene (15q11)**


(A)

(B)
Figure 3: Charles Dickens depicted an obese boy who has somnolence in “The Posthumous Papers of Pickwick Club”. Similar to a child with PWS, obesity, somnolence, small hands and feet are easily recognizable in the drawing.

At neonatal life the most striking features are hypotonia and poor feeding (Oiglane-Shlik, Zordania et al. 2006). The feeding problem can be due to hypotonia, poor suckling and swallowing. The neonate commonly needs gavage feeding for months. The other features suggestive of PWS are cryptorchism and small penis in males and hypoplastic labia in females (Stephenson 1980). Hypogonadotrophic hypogonadism is thought to be responsible for the signs. In an early study it was described that in boys the scrotal skin is not completely flat despite of cryptorchism and in girls the hypoplasia of labia is only apparent on careful inspection (Stephenson 1980).

The feeding problem usually resolves by 6 months of age and hyperphagia starts approximately from 18 months of age (Holm, Cassidy et al. 1993, Eiholzer 2005, Goldstone, Holland et al. 2008). Hypopigmentation is a common feature of PWS and it is reported to be present in about two thirds of the patients (Hittner, King et al. 1982, Butler 1989). Chromosome 15 controls the activity of enzymes responsible for melanin production and it is reduced in PWS leading to hypopigmentation. Poor growth in early childhood is also a feature of PWS (Butler and Meaney 1987). In a study, adult patients with PWS underwent growth hormone provocation tests with Arginine and growth hormone releasing hormone and they were found to have growth hormone deficiency, which may be responsible for the poor growth (Grugni, Marzullo et al. 2006). Global developmental delay
is also seen in children with PWS. However, a study reported that PWS patients scored better on visual motor discrimination skills than auditory verbal processing skills (Curfs, Verhulst et al. 1991). Behaviour problems in PWS can be a major issue and common behaviour phenotypes are tantrums, skin picking, obsessiveness, stubbornness, stealing and violence (Holland, Whittington et al. 2003). Another disabling problem in PWS is sleep-disordered breathing (SDB) (Arens, Gozal et al. 1994, Livingston, Arens et al. 1995, Nixon and Brouillette 2002, Yee, Buchanan et al. 2007, Camfferman, Doug McEvoy et al. 2008). SDB can be seen since early age but early oxygen therapy to infants with central hypoventilation and hypoxia appears to improve ventilation, central event index, and oxygenation (Urguhart, Gulliver et al. 2013). SDB leads to daytime hypersomnolence, respiratory failure and probable sudden death associated with growth hormone therapy (GHT) (Eiholzer, Nordmann et al. 2002, Craig, Cowell et al. 2006, Tauber, Diene et al. 2008). GHT has been shown to be beneficial in children with PWS. Numerous studies described the benefits of GH therapy in this syndrome (Davies, Evans et al. 1998, Carrel, Myers et al. 2002, Hoybye, Hilding et al. 2003, Angulo, Castro-Magana et al. 2007, Hoybye 2007, Myers, Whitman et al. 2007, Lindgren and Lindberg 2008, Mogul, Lee et al. 2008). GHT also improves the linear height and body composition in patients with PWS (Davies, Evans et al. 1998, Carrel, Myers et al. 1999, Davies 1999, Carrel, Myers et al. 2002, Eiholzer, L'Allemand et al. 2004, Bertella, Mori et al. 2007, Myers, Whitman et al. 2007, Lindgren and Lindberg 2008, de Lind van Wijngaarden, Siemensma et al. 2009). Children with PWS treated with GH (1 mg/m²/day) for 2 years demonstrated normalization of height standard deviation score, faster growth in head circumference, increased lean body mass accrual and decreased body fat together with improved language and cognitive functions (Myers, Whitman et al. 2007). In another study GH was continued for total of 4 years in three cohorts receiving different doses of GH. The benefit on growth velocity, body composition (lean body mass) and resting energy expenditure was noted with higher doses of GH (7 mg/m²/wk and 10.5 mg/m²/wk) but not with 2.1 mg/m²/wk dose (Carrel, Myers et al. 2002). Bone mineral density however improved in all studied doses of GH (Carrel, Myers et al. 2002). Moreover, GH improved sleep-related breathing disorders in children with PWS in a study in which 19 out of 25 patients had improvement in the Apnoea/ Hypopnoea Index (AHI) and Central Index (CI), but not of the Obstructive Index (OI) when polysomnography 6 months after commencement of GH was compared to that of baseline (Eiholzer, L’Allemand et al. 2004, Miller, Silverstein et al. 2006). One study examined the effect of commencing GHT early in childhood by analyzing data from the Ozgrow database of Australia and New Zealand regarding children with PWS on GH
therapy. The Australasian Paediatric Endocrine Group (APEG) established the Ozgrow database in an attempt to collect data pertaining to GH therapy in children in Australia and New Zealand. It was found that in children with PWS, there is better linear growth if GHT is started before three years of age than after three years (Nyunt, Harris et al. 2009). However, in a mortality review in patients with PWS, the majority of sudden death was related to respiratory pathology (Eiholzer, Nordmann et al. 2002, Tauber, Diene et al. 2008) and this finding was also supported by a study of the KIGS, Pfizer international Growth Database (Craig, Cowell et al. 2006).

Starting from 2008, the Commonwealth Government of Australia started to subsidize growth hormone therapy to all children who were genetically diagnosed of PWS until the age of 18 years. All children with PWS who are not severely obese (200% of ideal weight) receive growth hormone therapy (GrowthHormoneProgramme, DepartmentofHealth et al. 2014).

Patients with PWS have delayed onset of puberty in both males and females (Butler 1990, Eiholzer, Grieser et al. 2007). It is due to hypogonadotrophic hypogonadism. Sex steroids therapy not only induces pubertal changes but also improves bone mineral density (BMD) (Eiholzer, Grieser et al. 2007, Cassidy and Driscoll 2009). Central hypothyroidism is seen in 19% of patients with PWS (Sher, Bistritzer et al. 2002, Miller, Goldstone et al. 2008) and treatment with thyroxine is important for the neuronal, metabolic and bone health. One study reported very high prevalence of central hypoadrenalism in children with PWS using over-night Metyrapone test (de Lind van Wijngaarden, Otten et al. 2008). Subsequent studies, including our group from Brisbane, Australia, using low dose Synacthen test (Nyunt, Cotterill et al. 2010, Grugni, Beccaria et al. 2013), and a study using standard Synacthen test and insulin tolerance test (Farholt, Sode-Carlsen et al.), did not find such high prevalence of central hypoadrenalism in patients with PWS. Those studies prevented the use of physiological replacement or supra-physiological doses of corticosteroid during periods of stress.

PWS patients, during neonatal life and infancy, have feeding problem and failure to thrive (Cassidy and Driscoll 2009). The underlying cause for the feeding problem is thought to be poor sucking and swallowing related to muscular hypotonia. As a result there is failure to thrive. However, at an older age the appetite changes to insatiable form. The age of change in appetite varies in the current literatures. Some reported onset of weight gain is from 15 months to 4 years (Holm and Pipes 1976), 1 to 6 years (Butler 1990) and 10 to 18 months (Ehara, Ohno et al. 1993). After the change in appetite, the patients usually forage and often eat non-food items or discarded food. If they have free access to food, they do
not eat faster but longer than obese controls (Zipf and Berntson 1987, Lindgren, Barkeling et al. 2000). The fat mass is increased (up to 47%) compared to controls (Brambilla, Bosio et al. 1997, Goldstone, Brynes et al. 2002). The pathophysiology of the dimorphic nature of appetite and weight gain is unknown. It may be due to the differences in relation between ghrelin, acylated and des-acylated, and obestatin during young age and older age are responsible for dimorphic nature of appetite and weight gain.

Obesity becomes a major problem in adolescence age and it is believed to be due to well-described hyperghrelinaemia (Holland, Treasure et al. 1993, Lindgren, Barkeling et al. 2000, DelParigi, Tschop et al. 2002, Haqq, Farooqi et al. 2003, Butler and Bittel 2007). Although ghrelin stimulates appetite via Arcuate nucleus of the hypothalamus, the causal relation between ghrelin and hunger in PWS has not been established. The available evidence is higher blood ghrelin level is associated with higher subjective sense of hunger (DelParigi, Tschop et al. 2002). Reduced energy expenditure may also contribute to the development of obesity in PWS (van Mil, Westerterp et al. 2000, van Mil, Westerterp et al. 2000, Butler, Theodoro et al. 2007). Two cross sectional studies identified that adults with PWS had a number of orthopaedic problems such as developmental dysplasia of hips, scoliosis, genu vulgum, and ankle abnormalities (West and Ballock 2004, Kroonen, Herman et al. 2006). Those conditions hinder patients with PWS to be physically active. Other features of PWS include high pain threshold, inability to vomit, abnormal temperature regulation and acute abdomen due to gastric dilatation and necrosis (Cassidy and Driscoll 2009).

A number of studies examined the phenotypic difference in different genotypes of PWS. Patients with Type I deletion were found to have more behavioural and psychological problems than those with type II deletion or maternal UPD (Butler, Bittel et al. 2004). However, other studies did not find any difference in cognitive and behavioural phenotypes between two types of deletions (Milner, Craig et al. 2005, Varela, Kok et al. 2005). PWS patients with maternal UPD, are less likely to have typical facial features (Cassidy, Forsythe et al. 1997) and are more likely to have better verbal IQ (Roof, Stone et al. 2000).

1.2: PWS and Hypothalamic dysfunction
The hypothalamus is critical in regulating energy homeostasis by controlling hunger, satiation and energy expenditure in response to a variety of peripheral signals. Clinical features of PWS such as a combination of endocrine disorders; sleep-related breathing control disorder; defective temperature regulation; high pain threshold; inability to vomit;
hypotonia; developmental delay; hyperphagia; obesity and behavioural problems suggest that PWS may be due to defective Hypothalamus. Hypothalamus secretes releasing hormones which controls the production of pituitary hormones and deficiencies of such releasing hormones is believed to result in common endocrinological abnormalities of PWS. For example growth hormone secretions defect (Grugni, Marzullo et al. 2006), central hypothyroidism (Sher, Bistritzer et al. 2002) are due to the deficiency of growth hormone releasing hormone, and thyrotrophic releasing hormone respectively. Previously hypogonadism was thought to be due to hypogonadotrophic hypogonadism (Stephenson 1980) but recent reports suggest the primary gonadal failure is the major component of hypogonadism (Hirsch, Eldar-Geva et al. 2009, Eldar-Geva, Hirsch et al. 2010). Hirsch et al. reported that male patients with PWS had low to undetectable inhibin B, low testosterone and leutinising hormone. Eldar-Geva et al. reported that adult female PWS patients had low inhibin-B, low normal oestradiol and low leutinising hormone.

Sleep- disordered breathing (SDB) in PWS includes hypoventilation, reduced ventilatory response to hypoxia and hypercapnia, sleep- related hypoxaemia and obstructive sleep apnoea (OSA) (Nixon and Brouillette 2002). Apart from OSA, which may be related to obesity, the other SDB in PWS indicate hypothalamic regulatory defects. There are other abnormalities of sleep and arousal in PWS such as reduced rapid eye movement (REM) latency, sleep onset REM periods and cataplexy independent of obesity (Vela-Bueno, Kales et al. 1984, Kaplan, Fredrickson et al. 1991, Manni, Politini et al. 2001). Abnormal REM sleep indicates that the orexin control defect at hypothalamic level (Nevsimalova, Vankova et al. 2005). Furthermore, high pain threshold, inability to vomit and hyperthermia (Cassidy and Driscoll 2009) seen in PWS suggest abnormal hypothalamus.

1.3: Abnormal hypothalamus imaging in PWS
There are a number of studies using functional imaging that related PWS to a hypothalamic disorder (Shapira, Lessig et al. 2005, Hinton, Holland et al. 2006, Holsen,
In a functional Magnetic Resonance Imaging study, with the stimulus of high calorie diet, there was increased neuronal activation of appetite and motivation centers such as hypothalamus and Occipito-Frontal cortex in individuals with PWS than controls (Dimitropoulos and Schultz 2008). In another study using functional MRI on obese adult PWS patients, the lag time between oral glucose intake and activation of satiety centers of hypothalamus and other areas of brain is longer than that of obese controls (Shapira, Lessig et al. 2005). Measuring regional cerebral blood flow using positron emission tomography, PWS patients were also found to have absent pattern of neural activation associated with satiety even after a higher energy load but similar neural representation of hunger after overnight fasting (Hinton, Holland et al. 2006).

In another study using functional MRI, the PWS individuals had higher food-item-induced activity in limbic reward areas and lower activity at hypothalamus and hippocampus before a meal (Holsen, Savage et al. 2012). After a test meal, PWS individuals had higher activity in hypothalamus, amygdala and hippocampus but lower inhibitory activity in higher cortical regions. The authors concluded with the proof of functional MRI results that in PWS there is increased food reward and lack of cortical control of satiety. Hornea et al studied the grey and white matter volume, and their distributions in the brain in PWS comparing against healthy weight non-PWS controls (Honea, Holsen et al. 2012). Using a custom voxel-based morphometry processing stream, the authors showed PWS individuals had lower grey matter volumes in pre-frontal, orbitofrontal and temporal cortices, hippocampus and parahippocampal gyrus, and lower white matter volumes in the brain stem, cerebellum, medial temporal, and frontal cortex. Within PWS group, individuals with paternal deletion had lower grey matter volume primarily in the prefrontal and temporal cortices, and lower white matter in the parietal cortex. Those with maternal uniparental disomy had more extensive lower gray and white matter volumes in the orbitofrontal and limbic cortices compared to the control group. This may be the preliminary findings to explain the difference in the behavior phenotypes in different genetic subgroups of PWS.

1.4: Genetic explanation of hypothalamic dysfunction in PWS

Mage Like-2 (Magel 2) gene falls within PWS region, which is expressed mainly through paternal-specific allele, and its main function is to control the circadian rhythm by the hypothalamus. Magel-2 is highly expressed in Supra-chiasmatic nucleus, which is responsible for circadian rhythm through orexin signaling system (Peyron, Tighe et al.
Mice deficient in Magel-2 were found to have orexin signaling defect and therefore have abnormal circadian rhythm and reduced physical activity (Kozlov, Bogenpohl et al. 2007). Moreover, Magel 2 knockout mice show many features of PWS such as growth failure, hypogonadotropic- hypogonadism, and skin picking (Bischof, Stewart et al. 2007). Magel-2 null female mice displayed delayed and lengthened puberty while male mice had low testosterone and low gonadotropins. Both genders showed early reproductive decline and infertility (Mercer and Wewrick 2009). Necdin null mice have reduction in leutinising hormone releasing hormone and oxytocin neurones in hypothalamus as well as skin picking and improved visual memory similar to human PWS (Muscatelli, Abrous et al. 2000). Interestingly non-coding RNA, Snord116, in the PWS region was found to have expressions in para-ventricular and ventro-medial hypothalamus and arcuate nucleus (Zhang, Bouma et al. 2012). Two animal studies reported that paternally-derived Snord116 deleted mice had PWS phenotypes (Skryabin, Gubar et al. 2007, Ding, Li et al. 2008), and a case report claimed that deleted non-coding RNAs were responsible for all clinical features of PWS (Sahoo, del Gaudio et al. 2008). Those evidence supports that the underlying defect in PWS is at hypothalamus. Swabb et al. reported reduced number of oxytocin producing neurons in paraventricular nuclei in autopsy samples of PWS patients also suggested hypothalamic dysfunction (Swaab, Purba et al. 1995).

1.5: Hypothalamic control of Autonomic Nervous System (ANS)
ANS consists of parasympathetic (PSNS) and sympathetic nervous systems (SNS). It’s important function is to maintain homeostasis. The main neurons involved in ANS are preganglionic and postganglionic neurons. For SNS, preganglionic neurons are seen in intermediolateral cell column in the thoracic and lumbar (T1 to L2) segments of the spinal cord and ends at the paravertebral chain ganglia. Postganglionic neurons then commence from the ganglia and traverse a long path to end in the target organs such as heart, muscle vasoconstrictor, visceral vasoconstrictor, skin vasoconstrictor, skin vasodilator, sudomotor, pilomotor, pupilomotor, viseromotor units (Janig and Habler 2003, Guyenet 2006).
Parasympathetic outflow originates from the brain stem and sacral spinal cord. Unlike SNS, its ganglions are within or very close to its target organs. For example, the vagus nerve carries parasympathetic signals from medulla to target organs. Sacral component of parasympathetic output originates from lateral grey matter at S2 to S4 segments of sacral spinal cord (Birder, de Groat et al. 2010).
The central regulation mechanism of ANS involves the limbic system, which includes hypothalamus, certain brain stem regions particularly nucleus tractus solitarius and the spinal cord. Because of its important and extensive regulatory function, the hypothalamus is known as the main or head ganglion of the ANS. A study using a neuronal tracer cholera toxin B indicated that parasympathetic and sympathetic projection neurons intermingle in paraventricular nucleus (PVN) of hypothalamus and thus it controls both arms of ANS (Naito, Ohmori et al. 1994). Activation of different regions of the hypothalamus produces a variety of coordinated autonomic responses. For example, activation of dorsal hypothalamus increases the blood pressure, intestinal motility, and intestinal blood supply but decreases the blood supply to the skeletal muscles. However, activation of ventral hypothalamus increases in blood pressure and blood supply to the skeletal muscles but decreases intestinal motility and blood supply to the intestines (Thomas 2011).

Hypothalamus integrates afferent signals and discharges efferent output to target organs. For example, it receives afferent signals from the limbic system, which is responsible for emotion, and activates the efferent sympathetic nervous system which represents the classical “fight or flight” response described by Walter Cannon in 1929. Moreover, amygdala, hippocampus and prefrontal cortex receive associational information from the cortical and subcortical areas that are involved in higher sensory processing and memory. The output from these involves activation of ANS and hypothalamo-pituitary-adrenal axis (HPA) (Herman, Figueiredo et al. 2003).

Regulation of the circadian rhythm, sleep-wake cycle, thermoregulation, glucoregulation, osmoregulation, response to stress, and immunoregulation are controlled by hypothalamus and pre-optic area (Saper 2002, Thompson and Swanson 2003). The pre-optic area is functionally divided into three functional zones: periventricular, medial and lateral zones (Thompson and Swanson 2003). The periventricular zone includes supra-chiasmatic nucleus that controls circadian rhythm and pituitary gland. Medial pre-optic area orchestrates and coordinates autonomic and endocrine output for thermoregulation, osmoregulation and stress response (Sawchenko, Li et al. 2000, Yoshida, Li et al. 2009). The paraventricular nucleus, dorso-medial hypothalamus and lateral hypothalamic area are main regions for ANS output (Saper 2002).

The brain stem acts as the relay station and also regulates ANS. Peri- aqueductal gray matter, para-brachial nucleus, some areas of pons and medulla including nucleus tractus solitarius and medullary raphe receive converging visceral and somatic information; reciprocally interconnect with the hypothalamus and spinal tracts; and generate stimulus-
specific patterns of autonomic, endocrine and motor responses (Cersosimo and Benarroch 2013). Medulla oblongata and Pons are crucial for tonic and reflex regulation of arterial pressure via the spinal sympathetic outflow (Alexander 1946, Dampney 1981). The neurons in the nucleus tractus solitarius are where afferent signals from baro- receptors terminate (Reis, Granata et al. 1984). Those neurons maintain tonic background drive to sympathetic preganglionic neurons directly (Dampney 1981, Reis, Granata et al. 1984); and are the target organs for the centrally acting anti- hypertensive agents such as Clonidine (Haeusler 1973). A1 fibers from the Nucleus Tractus Solitarius project cephalically to the paraventricular and supraoptic nuclei of hypothalamus for integration of sensory signals (Blessing, Goodchild et al. 1981, Blessing, Jaeger et al. 1982). On the other hand, C1 fibers of the NTS projects caudally to the spinal cord (Ross, Ruggiero et al. 1984). The intermediolateral column of the spinal cord innervates adrenal medulla and sympathetic ganglia (Reis, Granata et al. 1984). The gamma amino- butyric acid (GABA) is the main neurotransmitter for the NTS- C1 projection (Yamada, Norman et al. 1982). The vagus nerve means wandering in Latin and is the longest cranial nerve. It arises from medulla and traverses a long path from the cranium to the neck, thorax, and abdomen (Ruffoli, Giorgi et al. 2011). It is formed from the 4th branchial branch and comprises mostly sensory fibers carrying visceral signals from head, neck and thorax (Agostoni, Chinnock et al. 1957). The primary sensory input of vagus nerve is mostly from baro- receptors and chemoreceptors from the heart and aortic arch. Those afferent fibers terminate at the NTS which further co- ordinates sympathetic and parasympathetic discharges (Nieuwenhuys, Voogd et al. 2008). A short pathway connects NTS to dorsal motor nucleus of vagus in discharging parasympathetic output (Nieuwenhuys, Voogd et al. 2008). The fibers arising from dorsal motor nucleus of vagus descend and terminate in parasympathetic ganglia which are in close proximity to the target organs (Jean 1991, Travagli, Hermann et al. 2006). Different parts of dorso- motor nucleus of vagus have parallel stimulatory and inhibitory pathways on gastrointestinal motility (Chang, Mashimo, & Goyal, 2003). Most of the inhibition induced by NTS- dorsal motor nucleus of vagus is mediated by GABA acting on the GABA-A receptor (Travagli, Gillis et al. 1991). Conversely, the excitatory action is mediated by glutamate on N- methyl D- aspartate (NMDA) receptors and non- NMDA receptors (Willis, Mihalevich et al. 1996). Tonic fashion of GABAergic output from NTS to dorsal motor nucleus of vagus has an impact on vagal reflex control of its target organs (Travagli, Hermann et al. 2006). (Figure 4)
Figure 4: ANS reflex Arc and its connections to hypothalamus via nucleus tractus solitaries. IX- Glossopharyngeal nerve, X- Vagus nerve, C1- C1 fibers of medulla oblongata, DMV- dorsal motor nucleus of vagus nerve, GABA- glutamic acid dehydrogenase
1.6: Hypothalamus and ANS in PWS

Since PWS is considered to be a hypothalamic dysfunction, the control of ANS in PWS is likely to be defective. There is one case-control study that reported that there is a detectable ANS dysfunction in patients with PWS (DiMario, Dunham et al. 1994). In the study participants had measurements of simultaneous and serial electrocardiogram (ECG), pulse rate and blood pressure during lying and standing and plasma norepinephrine during lying and standing. The pupillary response to topical Pilocarpine (pupillary constrictor acting on muscarinic receptors of parasympathetic nervous system) was also examined to complement the cardiovascular response to physiological stimulation of ANS. In PWS cohort, there was less pronounced change in diastolic blood pressure after standing, and higher pulse rate in those with greater BMI. PWS participants had trend towards lower diastolic blood pressure. Pupillary constriction of 2 mm or more with topical Pilocarpine is noted in half of the PWS cohort but not in the controls, and the 30:15 R-R interval ratio on the ECG was abnormal in 6 out of 14 PWS participants. The authors concluded that there was detectable dysfunction of ANS in PWS patients particularly in diminished parasympathetic activity. When dissecting further on the results of this study, poor homeostatic regulation of diastolic blood pressure on standing in PWS cohort suggested sympathetic failure to vasoconstrict when the blood column dropped because of gravity and baro-receptors were stimulated. Pilocarpine is a parasympathomimetic and in normal individuals with intact muscarinic receptors on pupillary smooth muscles, it constricts pupils. In the study, none of the control participants had pupillary constriction but 50% of the PWS group did. It indicated that the muscarinic receptors in normal controls were not responsive but only 50% of PWS group were to Pilocarpine. The effect of Pilocarpine did not indicate any central ANS actions. DiMario et al also reported in a case-control study that PWS patients had lower average temperature and lower temperature trend suggesting poor sympathetic function (DiMario and Burleson 2002). The same group of authors further examined the parasympathetic function by respiratory sinus arrhythmia and found that the PWS group had less variable heart rate with different phases of breathing indicating poor parasympathetic outflow (DiMario, Bauer et al. 1993).

Purtell et al also studied ANS function in PWS. In the study Purtell et al used SphygmoCor (AtCor Medical, Sydney, NSW, Australia) to measure heart rate variability and arterial stiffness in adults with PWS and healthy obese controls (Purtell, Jenkins et al. 2013). The participants were then given a mixed meal to stimulate ANS function and the measurements were done before the meal and at 30, 60, 120, 180 and 240 minutes after
the meal. The PWS group displayed similar high frequency heart rate variability after a meal to the control group suggesting parasympathetic function is not different to the controls after a meal. The low frequency responsiveness, which is dependent upon both sympathetic and para-sympathetic functions, was reduced in PWS group.

Wade et al. also examined the cardiac autonomic modulation in a case-control study and baro-reflex sensitivity (Wade, De Meersman et al. 2000). In the study, electrocardiogram, blood pressure, heart rate and respiration were recorded in supine, after transition from supine to standing, sitting, during a Valsava maneuver, while performing moderate exercise, and during recovery from exercise in sitting position. The study did not find any difference in cardiac autonomic modulation (Wade, De Meersman et al. 2000). The above studies examined a single target organ, the heart, which has dual ANS innervations. The activation and inhibition of both sympathetic and para-sympathetic systems on heart occur quickly and frequently at times; and slowly and in sustained fashion at another times (Saul, Albrecht et al. 1988, Thomas 2011). For this reason it may be very difficult to study the ANS function on a single organ at a single time point.

The higher pain threshold that is commonly seen in PWS may be attributed by diminished ANS action on cutaneous nerve complexes and pain receptors via GABA system (Hoefnagel, Costello et al. 1967, Bray, Dahms et al. 1983). Selective activation of GABA-B receptor by baclofen produced anti-nociception, that is increased pain threshold (Hill and Bowery 1981, Sawynok and Dickson 1983). Reduced salivation also suggests paucity of parasympathetic nervous outflow in PWS (Hoefnagel, Costello et al. 1967). PWS patients have abnormal temperature regulation and it may be due to defective regulation of ANS (Bray, Dahms et al. 1983).

1.7: Ghrelin
Ghrelin has growth hormone releasing activity as it is thought to be an endogenous ligand of the growth hormone secretagogue receptor (Kojima, Hosoda et al. 1999). Ghrelin is a peptide of 28 amino acids secreted from the fundus of the stomach. N-octanoylation by the enzyme ghrelin O-acyl transferase (GOAT) (Gutierrez, Solenberg et al. 2008, Yang, Brown et al. 2008) at serine-3 residue of ghrelin molecule to form acylated ghrelin which is an active form of ghrelin (Kojima, Hosoda et al. 1999). In human, the prominent form of circulating ghrelin is des-acyl form (2.5 folds higher than acyl ghrelin) (Broglio, Gottero et al. 2004) and it was initially considered to be an inactive form of ghrelin (Hosoda, Kojima et al. 2003). Active ghrelin acts on the G-coupled receptors with specific ligand which are present largely in pituitary and hypothalamus (Gnanapavan, Kola et al. 2002). The
receptors are also seen in other parts of the brain which are involved in control of bi- 
rhythms, mood, cognition, memory and learning (van der Lely, Tschop et al. 2004). In 
addition, they are also reported to be present on multiple organs such as intestine, 
kidneys, heart, adrenal glands, pancreas, thyroid and gonads (Gnanapavan, Kola et al. 
2002).

Ghrelin molecule derives from prohormone preproghrelin which consists of 117 amino 
acids. Other peptides called obestatin and desacyl ghrelin also derives from preproghrelin 
(Kojima, Hosoda et al. 1999, Delhanty, Neggers et al. 2012). While ghrelin is orexigenic, 
obestatin is anorexigenic although both peptides derive from the same parent molecule. 
Rats treated with obestatin had reduced appetite, weight and jejunal motility (Zhang, Ren 
et al. 2005).

At Arcuate nucleus of hypothalamus ghrelin increases expression of neuropeptide Y (NPY) 
and Argouti Related Peptide (ArgRP) which increase short- term and sustained appetite 
respectively (Nakazato, Murakami et al. 2001). Desacyl ghrelin was previously thought to 
be an inactive peptide (Hosoda, Kojima et al. 2003) but current understanding suggests 
the opposite (Delhanty, Neggers et al. 2012). It is believed to be an independent hormone 
and it rapidly modulates the expression of metabolically important genes in adipose tissue, 
muscles and liver (Delhanty, Sun et al. 2010). Desacyl ghrelin also acts on growth 
hormone secretagogue receptor and only in supra physiological concentration it displaces 
acyl ghrelin (Gauna, van de Zande et al. 2007). The antagonistic action against acyl 
ghrelin was supported by another study (Broglio, Gottero et al. 2004). In the study co- 
administration of acyl and desacyl ghrelin abolished acyl ghrelin- induced change in 
plasma insulin and glucose. Increased acyl and desacyl ghrelin ratio is associated with 
obesity and type 2 diabetes mellitus (Barazzoni, Zanetti et al. 2007, St-Pierre, Karelis et al. 
2007). Plasma insulin and HOMA-IR were negatively correlated with desacyl ghrelin but 
positively with acyl ghrelin and acyl to desacyl ghrelin ratio (Barazzoni, Zanetti et al. 2007). 
Vagus nerve is thought to be important in controlling ghrelin secretion. Vagus nerve 
experts tonic suppression of ghrelin production as higher blood ghrelin level was found in 
vagotomised mice (Lee, Wang et al. 2002). Furthermore, in an animal study the plasma 
ghrelin level was suppressed after electrical stimulation on the vagus nerve (Murakami, 
Hayashida et al. 2002). Increased ghrelin level is seen during fasting when the vagal 
action is at a nadir.

Physiologically there is pre-prandial rise and postprandial fall of ghrelin associated with a 
meal (Cummings, Purnell et al. 2001). The pre-prandial rise of the level is thought to be 
due to the cephalic phase of the digestive process, modulated by the efferent vagus nerve.
The rise of plasma ghrelin during fasting was less pronounced in vagotomised rats compared to sham operated controls before feeding (Williams, Grill et al. 2003). In human studies cephalic phase stimulation of food via vagal efferent fibers by sham feeding was found to be associated with rise in ghrelin before meal and with more rapid fall in postprandial period (Robertson, Jackson et al. 2001, Arosio, Ronchi et al. 2004, Bizzarri, Rigamonti et al. 2004, Heath, Jones et al. 2004). In healthy human subjects, the plasma ghrelin level was significantly lower after a sham feeding (chew and spit) than that of those who had a standard mixed meal (Arosio, Ronchi et al. 2004). Sham feeding has been proven to be an inducer for parasympathetic nervous outflow (Robertson, Jackson et al. 2001).

The physiological post-prandial fall is believed to be due to vago-vagal inhibition (Robertson, Jackson et al. 2001, Arosio, Ronchi et al. 2004, Bizzarri, Rigamonti et al. 2004, Heath, Jones et al. 2004). From those literatures it has been postulated that parasympathetic nervous system suppresses blood ghrelin level tonically and at postprandial period.

1.8: Nesfatin-1

First information of Nucleobindin 2 (NUCB2) - encoded satiety and fat- influencing protein 1 (nesfatin-1) was provided in 2006 (Oh, Shimizu et al. 2006). It inhibits food intake upon injection to ventricles in animal studies (Oh, Shimizu et al. 2006, Shimizu, Oh et al. 2009, Purtell, Jenkins et al. 2013). Only the Mid- segment of nesfatin-1 contains more potent anorexigenic effect compared to N- segment or C- segment of Nesfatin-1 (Shimizu, Oh et al. 2009). The anorexigenic effect of mid- segment of nesfatin-1 is independent of leptin pathway as reduced food intake was still observed in db/db mice and overfed obese mice (Shimizu, Oh et al. 2009, Purtell, Jenkins et al. 2013). Central injection of nesfatin-1 increases c-fos expression in Paraventricular nucleus of hypothalamus and Nucleus Tractus Solitarius (Young, Deo et al. 2010, Purtell, Jenkins et al. 2013). It indicated that Nesfatin-1 acts on hypothalamus and NTS.

In another animal study using immunohistochemistry, co-localization of ghrelin and nesfatin-1 was observed in both hypothalamus and anterior intestine of the goldfish (Young, Deo et al. 2010). In the same study, intracerebroventricular injection of nesfatin-1 and ghrelin reduced the mRNA expression of pre-proghrelin and NUCB2 in hypothalamus respectively indicating reverse relationship between ghrelin and Nesfatin-1 (Young, Deo et al. 2010).
Maejima et al. elegantly described that nesfatin-1 regulates oxytocinergic signaling in hypothalamus and Nucleus Tractus Solitaries which in turn stimulates melanocortin pathway to induce anorexia independent of leptin (Purtell, Jenkins et al. 2013). To date, little is known about plasma profile of nesfatin-1 in relation to a meal in healthy human or nesfatin-1 status in PWS where satiety is not commonly achieved after a meal.

1.9: PWS and hyperghrelienaemia
In PWS, energy gain is increased by hyperphagia and the satiation cannot be effectively achieved after food intake (Holland, Treasure et al. 1993). In the case-control study hyperphagia in PWS was clearly demonstrated by using cucumber sandwiches. High Ghrelin level is found in PWS and it is believed to be responsible for hyperphagia and poor satiation in PWS (DelParigi, Tschop et al. 2002, Haqq, Stadler et al. 2003, Haqq, Grambow et al. 2008). Higher fasting plasma ghrelin level and denser ghrelin secreting cells in stomach were seen in PWS than control group (Haqq, Farooqi et al. 2003, Choe, Song et al. 2005, Haqq, Grambow et al. 2008). However, the direct link between high ghrelin level and increased appetite has not been established to this stage. DelParigi et al. reported that there was positive correlation between plasma ghrelin level and subjective ratings of hunger (DelParigi, Tschop et al. 2002).

A number of studies reported failure of suppression of plasma Ghrelin after a meal but an uncontrolled study demonstrated lower plasma Ghrelin after a meal than before a meal in patients with PWS (DelParigi, Tschop et al. 2002, Haqq, Stadler et al. 2003, Paik, Choe et al. 2006, Gimenez-Palop, Gimenez-Perez et al. 2007). Therefore hyperphagia in PWS may be due to high and unregulated ghrelin secretion, which may be due directly to low, or loss of tonic vagal suppression, and lack of post-prandial vago-vagal suppression of ghrelin respectively. Alternatively it may also be indirectly due to low nesfatin-1 as a result of dysfunctional ANS in PWS.

1.10: Hypothalamic control of energy homeostasis
Arcuate nucleus (ARC) of hypothalamus integrates information conveyed by different peripheral signals of energy homeostasis. It produces Neuropeptide Y (NPY) and agouti-related protein (Agrp) which are orexigenic peptides. These neuropeptides are associated with central control of appetite as their expression increase at the time of maximal spontaneous feeding in an animal study. NPY is a 36 amino acids peptide similar to pancreatic polypeptide family (Tatemoto, Carlquist et al. 1982). In an animal study, injection of NPY into cerebral ventricles leads to increased food intake (Clark, Kalra et al. 2014).
NPY stimulates Argp, which increases sustained food intake (Hagan, Rushing et al. 2000). Another animal study showed that when rats were treated with intracerebroventricular injections of ghrelin, NPY and Argp there was significant reduction in locomotor activity of the animals compared to the controls that received vehicle (Tang-Christensen, Vrang et al. 2004). As animals do not have volitional exercise, the reduction in locomotor activity can be considered as non-exercise associated thermogenesis.

Lateral hypothalamus contributes to food intake via orexin and melanin- concentrating hormone (MCH) although the stimulating effect of food intake in this pathway is short lived (Sakurai, Amemiya et al. 1998). Orexin cells sends projections to ARC and they form synapses with NPY and Argp cells which in turn synapse back to orexin cells (Horvath, Diano et al. 1999). There are two groups of orexin neuropeptide, orexin A and orexin B (Willie, Chemelli et al. 2001). The peptides act on orphan G- coupled- receptor protein (Sakurai, Amemiya et al. 1998). Orexin secreting neurones are seen in lateral hypothalamic and perifonical area (Peyron, Tighe et al. 1998, Sakurai, Amemiya et al. 1998, Chemelli, Willie et al. 1999). Its neurones are projected throughout central nervous system to nuclei that control feeding (Kotz 2006), sleep- wakefulness, neuroendocrine functions and autonomic regulation (van den Top, Nolan et al. 2003). Orexin knockout mice showed sleep- onset Rapid Eye Movement (REM) period, sleep fragmentation and reduced wakefulness compared to wild littermates (Hara, Beuckmann et al. 2001). Targeted disruption of orexin gene in mice resulted in phenotype similar to human and canine narcolepsy (Willie, Chemelli et al. 2001). The sleep- related breathing disorders in PWS are also similar to orexin deficiency and it is postulated that PWS is a state of orexin deficiency as part of hypothalamic lesion (Nevsimalova, Vankova et al. 2005). In a rat study, intra- cerebroventricular injection of orexin was associated with wakefulness and increased locomotor activity (Kiwaki, Kotz et al. 2004).

ARC also has anorexigenic function through neurons that produce α- melanocyte stimulating hormone (α-MSH), which derives from cleaving of pro-opiomelanocortin (POMC) protein (Cone, Cowley et al. 2001). It binds the melanocortin receptors 3 and 4 (MC3R/ MC4R) to reduce food intake (Boston, Blaydon et al. 1997). Moreover, POMC cells synthesize another anorexigenic peptide called cocaine and amphetamine related transcript (Elias, Lee et al. 2001). Insulin, pancreatic hormone, and leptin, an adipokine, increases α-MSH and decreases NPY and Argp activities in ARC of the hypothalamus resulting in reduced food intake and increased energy expenditure.
There is a number of gastro-intestinal and other meal related hormones or peptides that act on the hypothalamus via vagus nerve or cervical sympathetic nerves. They include cholecystokinin, glucagon-like peptide 1, insulin, pancreatic peptide Y, apolipoprotein A-IV, enterostatin, bombesin, oxyntomodulin, and amylin which are considered to be satiation signals.

Adiposity signal, leptin, on the other hand acts on POMC and MC 3/4R system at ARC increases energy expenditure. Leptin deficient ob/ob mice had increased oxygen consumption after treatment with leptin (Pelleymounter, Cullen et al. 1995). The ob/ob mice have lower energy expenditure than their control littermates suggesting leptin is important for energy expenditure (James, Davies et al. 1978). Insulin is another adiposity signal and when injected into paraventricular nuclei (PVN) of hypothalamus, it increased energy expenditure without increasing locomotor activity (Menendez and Atrens 1991). Similarly, microinjection of CART to ARC resulted in increased locomotion (Abbott, Rossi et al. 2001). In an animal study, mice with mutation of MC4R showed reduced locomotor activity and oxygen consumption compared to the wild type mice (Ste Marie, Miura et al. 2000). PVN also mediates locomotor activity and energy expenditure by modulating sympathetic nervous outflow (Atrens and Menendez 1993). Thyrotropin releasing hormone produced by the hypothalamus also contributes to energy expenditure by stimulating thyroid stimulating hormone and thyroid hormone (Yarbrough 1979, Lechan and Fekete 2006).

After integration of the afferent peripheral signals in many parts of hypothalamus, efferent effector homeostatic mechanism ensues which include arousal, vigilance, physical activity, interaction with motor and autonomic nervous systems (Jones 2003).

1.11: Energy Expenditure (EE)

Total energy expenditure (TEE) can be generally divided in Basal Metabolic Rate, Activity related thermogenesis, energy cost of activity (AAT), and Thermic effect of food (TEF). Because thermic effect of food intake is negligible, energy expenditure is dependent upon Basal Metabolic rate and Activity thermogenesis (Levine 2004, Levine 2004). Basal metabolic rate (BMR) is the energy required for core body function and is measured at rest without food (Daan, Masman et al. 1989). Resting energy expenditure (REE) is defined as daily energy used during resting. BMR or REE usually accounts for approximately 60% of the daily energy expenditure (Ford 1984). Thermic effect of food is the energy used in digestion, absorption and storage of nutrients (Hill, DiGirolamo et al. 1985, Donahoo, Levine et al. 2004). It usually accounts for 10% of total energy usage. Levine further
divides activity related thermogenesis into exercise-associated (EAT) and non-exercise associated thermogenesis (NEAT) that he defines as energy expenditure related to activity other than volitional exercise (Levine, Schleusner et al. 2000). NEAT includes fidgeting, chewing gum, pacing around a room, toe tapping, shopping, dancing, gardening and so on. In industrialized countries the non-exercise associated thermogenesis is the most important component of total daily energy expenditure. Even in avid exercisers, non-exercise associated thermogenesis is the most predominant component of total daily energy expenditure. Low non-exercise associated thermogenesis due to mechanization of day to day life can lead to obesity (Levine 2007).

1.12: EE in PWS
In patients with PWS, unadjusted total energy expenditure, resting metabolic rate, and activity associated thermogenesis were found to be low in a study (Butler, Theodoro et al. 2007). Total energy expenditure and resting metabolic rate after correcting for fat mass remained significantly different. The authors related low metabolic rate to reduced lean body mass. However, the duration of the study was only 8 hours on each participant and the result may not represent the actual EE and EE was adjusted to neither weight nor fat free mass. Other studies found that resting and basal metabolic rates in patients with PWS are higher than the controls after having adjusted for the lean body mass (van Mil, Westerterp et al. 2000, Goldstone, Brynes et al. 2002). Obesity and relatively higher RMR suggests that the activity associated energy expenditure must be definitely low in patients with PWS. EAT may be insignificant because patients with PWS rarely exercise (Davies and Joughin 1993, van den Berg-Emons, Festen et al. 2008). Therefore NEAT represents AAEE in PWS and it is very likely to be low. ANS dysfunction and probably hyperghrelinaemia may be the causes of low NEAT in PWS.

1.13: Measuring EE
Measuring metabolic rates in children with PWS is made difficult by behaviour problems and current available methods of measuring metabolic rate may not be suitable as they can be cumbersome, intimidating and may cause claustrophobia. There is another device called Fitmate Pro that measures oxygen consumption in expired air to calculate metabolic rates. It is small, portable and less intimidating. Fitmate Pro has a facial mask that covers mouth and nostrils, which makes it a suitable alternative for measurement of metabolic rates and has been validated for the use for adults (Nieman, Austin et al. 2006, Nieman, Lasasso et al. 2007) but not for children.
Accelerometer is a device that is used to measure frequency and intensity of movement of human body. It is a uniaxial accelerometer that measures acceleration in vertical plane and calculates activity associated metabolic rate from the measurements. Actiheart is an accelerometer that measures uni-directional movement and heart rate to calculate metabolic rates. It is the first device that combines a heart rate monitor and accelerometer in a single unit. The main component of it is 6 mm thick with a diameter of 33 mm and houses a movement sensor, a rechargeable battery, a memory chip and other electronics. A wire approximately 100 mm in length runs to a smaller electronic chip. The total weight is only 10 gm. It measures heart rate variability and ECG amplitude for a set time resolution. It does the measurements in epochs, which is 15s, 30s and 1 min. The memory capacity of 128 kb allows the data to be stored for more than 11 days with an epoch setting of 1 minute.

Acceleration is measured by piezoelectric element contained in the Actiheart with a frequency range of 1-7 Hz. This movement sensor generates a transient charge when exposed to acceleration and produces a voltage signal which in turn gets converted into a binary signal by 8 bit analog to digital converter. This results in 256 distinctive levels of acceleration (128 positive and 128 negative levels). Acceleration is quantified as a numerical difference from zero acceleration in binary units. The binary units are stored and summed up over the epoch. The sensitivity of Actiheart in measuring heart rates is 0.250 mV. At the end of an epoch, mean R-R intervals of ECG is calculated and is converted to beats per minute.

1.14: Reasons for performing this study

Sinnema et al. in 2012 reported that the average life expectancy of individuals with PWS has increased over the recent years. In a cross sectional study published in 2011 by Sinnema et al., it was reported that individuals with PWS who are older than 50 years of age had cardiovascular disorders such as type 2 diabetes mellitus (50%), hypertension (25%), and stroke (25%) (Sinnema, Maaskant et al. 2011, Sinnema, Schrander-Stumpel et al. 2012). The authors reported early day-to-day functional decline in older PWS patients but not on the prevalence of ischaemic heart disease. Another study on PWS patients with mean age of 28 years showed they had higher high-sensitivity C-reactive protein, less exercise capacity, and greater microvascular dysfunction, that are associated with coronary artery disease (Patel, Harmer et al. 2007). In an adult PWS study, both visceral and subcutaneous adipose tissue increased with increasing age (Tanaka, Abe et al. 2013). The adiposity was low among adult PWS patients while continuing growth hormone
therapy, but it increased with age to similar levels to untreated patients once growth hormone was ceased (Tanaka, Abe et al. 2013). In obese subgroup of adult PWS patients, adiponectin, which is cardio-protective (Bluher and Mantzoros 2015), was low as visceral adiposity increased, and was negatively correlated with total cholesterol, low-density lipoprotein and triglyceride (Tanaka, Abe et al. 2013). During childhood the metabolic phenotype in PWS is more favourable compared to obese controls (Haqq, Muehlbauer et al., Brambilla, Crino et al. 2011). Even in early adult life, PWS patients maintain such favourable metabolic status (Talebizadeh and Butler 2005, Grugni, Crino et al. 2013, Lacroix, Moutel et al. 2014). As they get older, the adiposity and microvascular dysfunction increase to a critical level. High-energy gain and low energy expenditure may be the underlying cause of increased adiposity and obesity in PWS. Therefore it is very important to understand factors controlling the energy balance in PWS. To understand better the gaps in knowledge about PWS we performed this study to examine increased energy intake and decreased energy expenditure in PWS. Increased energy intake is believed to be related to high blood ghrelin level which may be due to poor ANS function in PWS as explained before. We therefore aimed to study acyl ghrelin profile related to a mixed meal, complete ANS functions and the correlation of two. Decreased energy burning may be at different modalities of thermogenesis and we aimed to measure all aspects of energy expenditure in PWS. Because hypothalamus controls energy usage through ANS, we aim to correlate energy expenditure to ANS output.

1.15: Hypotheses
Obesity in PWS is believed to be due to impaired energy homeostasis at hypothalamus level where there is imbalance in energy intake and energy expenditure. Hyperphagia in PWS is the cause of increased energy intake and it is thought to be related to high and unregulated ghrelin level seen in PWS, which drives insatiable appetite. ANS has a role in controlling ghrelin production during fasting and post-prandial periods. It is my hypothesis that, dysautonomia, as part of hypothalamic lesion in PWS, leads to abnormal ghrelin suppression. Furthermore, I hypothesize that the total EE and NEAT are reduced in PWS. Hypothalamus controls NEAT through ANS outflow. NEAT is dependent upon ANS, wakefulness, and ghrelin status. It is biologically possible that NEAT is low in patients with PWS. Hence, it led to my hypothesis that the total EE and NEAT are lower in PWS than the controls. The effect of low TEE and NEAT cannot be compensated by relatively higher fat free mass adjusted REE as previously reported (van Mil, Westerterp et al. 2000, Goldstone, Brynes et al. 2002).
Chapter 2: ANS function and acyl ghrelin in children PWS

2.1: Introduction

ANS in PWS

Autonomic nervous system has been shown to control plasma ghrelin level as described in the introduction section of the thesis.

A number of studies reported that there is likely to be ANS dys-function in PWS (DiMario, Bauer et al. 1993, DiMario, Dunham et al. 1994, DiMario and Burleson 2002, Purtell, Jenkins et al. 2013). Di Mario et al. performed a case-control study (DiMario, Dunham et al. 1994) and demonstrated lower resting diastolic blood pressure and lower change of orthostatic diastolic blood pressure in children with PWS. In the study, seven participants out of fourteen with PWS had pupillary constriction after Pilocarpine topical eye drops but none of the controls did. However, the results of measures of autonomic functions were inconsistent. Lower resting diastolic pressure and lower orthostatic change in blood pressure suggest poor sympathetic function and absence of pupillary constriction in fifty percent of the PWS participants suggest that local muscarinic acetyl choline receptors are defective rather than the central parasympathetic failure. A case-control study of PWS and controls (DiMario and Burleson 2002) did not find any difference in cutaneous blood flow as a response to change in body temperature. In another case-control study of PWS and controls, PWS patients had sinus arrhythmia with higher heart rate but less variability than controls (DiMario, Bauer et al. 1993).

Purtell et al. also studied ANS function in PWS and described findings indicating poor ANS function in PWS. The study used SphygmoCor (AtCor Medical, Sydney, NSW, Australia) to measure heart rate variability and arterial stiffness in adults with PWS and healthy obese controls (Purtell, Jenkins et al. 2013). The participants were then given a mixed meal to stimulate ANS function and the measurements were repeated at 30, 60, 120, 180 and 240 minutes after the meal. The study found that the parasympathetic function in PWS group was indifferent to the controls but low frequency heart rate variability, which is dependent upon both parasympathetic and sympathetic outflow, was reduced. The authors therefore assumed that sympathetic function might be suboptimal in PWS. The study examined a single target organ, the heart which has dual ANS innervation. The activation and inhibition of sympathetic and para-sympathetic systems occur very quickly and frequently at times; and slowly and in sustained fashion at another times (Saul, Albrecht et al. 1988, Thomas 2011). For this reason it may be very difficult to study the
ANS function on a single organ at a single time point.

Therefore, more data on ANS function in PWS may fill the gap in knowledge which in turn may result in possible treatment for PWS.

**Autonomic nervous control on orthostatic blood pressure**

Changing position from lying to standing normally increases the heart rate and systolic blood pressure within seconds followed by fluctuating decline thereafter even below the resting supine measurement (Borst, Wieling et al. 1982, Borst, van Brederode et al. 1984). The diastolic pressure behaves similarly but it reverts back to that of supine position after maximum heart rate has been achieved (Borst, Wieling et al. 1982, Borst, van Brederode et al. 1984). The effect of standing from the supine position is augmented by a preceding rest but not influenced by physical training. Compression of the blood vessels by the contracting postural muscles such as calf muscles leads to immediate rise in blood pressure. Acceleration of heart rate on rising is a reflex as active muscle contraction stimulates sympathetic nervous system and inhibits parasympathetic nervous system (Borst, Wieling et al. 1982, Borst, van Brederode et al. 1984).

The drop in blood pressure after standing is sensed by the baro- receptors at the Carotid sinus and the heart that carry the signals to Nucleus Tractus Solitarius by Glossopharyngeal and Vagus nerves respectively. The neurotransmitter involved is L-glutamate. NTS relays signals from glossohypopharyngeal and vagus nerves to PVN and SON of hypothalamus via A1 fibers and to the thoracic spinal cord via C1 fibers. The neurons of the NTS connect to C1 fibers by means of neurotransmitter GABA. The C1 neurons project to the intermediolateral and intermediomedial levels of the spinal cord, which then relay to either adrenal medulla or sympathetic ganglion where noradrenergic and adrenergic post-ganglionic neurons arise to innervate target organs such as blood vessels and cardiac muscles (Andersson, Cabero et al. 1996). (Figure 4) Hence orthostatic stimulation is a way to assess sympathetic nervous function (Hall 1988).

**Post-prandial ANS stimulation**

There have been a number of publications on the Sympathetic nervous stimulation after a meal. In a descriptive study, eight healthy young participants had radio- nucleotide cardiography in supine position before and after a standard meal. The meal increased the stroke volume and ejection fraction indicating positive inotropic and chronotropic effects of a meal. The study also showed there was dilation of left ventricle appreciably. It indicated
stimulation of sympathetic nervous outflow (Kelbaek, Munck et al. 1989). The same group studied thirty-six patients who recently had the first acute myocardial infarction (Kelbaek, Gjorup et al. 1989). After one month from hospital discharge, the participants who had acute myocardial infarction were randomized to meal and no meal groups. On supine position, all subjects had heart rate monitored by three-lead electrocardiogram, mean blood pressure determined by auscultation method, cardiac output measured by radionuclide cardiography, and venous plasma catecholamine measurement. There were no significant haemodynamic changes in those who had no meal but increased stroke volume; higher heart rate and cardiac output were noted in those who had a standard breakfast.

Scott et al. reported that carbohydrate ingestion increased muscle sympathetic nerve activity and calf blood flow (Scott, Greenwood et al. 2013). In the study, the authors showed that after carbohydrate ingestion, sympathetic nervous outflow was even higher in subjects with congestive heart failure than controls. Patients with congestive heart failure are considered to have higher sympathetic activity. Cox et al. (Cox, Kaye et al. 1995) studied normal healthy and lean participants on the meal effect on Sympathetic nervous function. After a meal, the Peroneal nerve sympathetic nerve activity increased from 7.7 burst per minute to 17.9 bursts per minute and the total body noradrenaline spill over from neurons correspondingly increased from 24% to 56%. Another group (Sidery, Cowley et al. 1993) also studied 10 healthy individuals by giving high carbohydrate meal and high fat meal. The authors showed that the sympathetic nervous system was stimulated after both high carbohydrate and fat meals. Cardiac output increased by 32% and 22% above the pre-meal state in the participants after a high carbohydrate meal and a high fat meal respectively. Fifteen minutes after high carbohydrate intake, the blood flow in superior mesenteric artery also rose by 87% and by 122% after high fat intake. In another study (Ryan, Goldberger et al. 1992), the authors described post-prandial sympathetic activation. The authors compared the cardiovascular response to a meal in young healthy individuals and ambulatory elderly individuals. The authors assumed there was blunting of sympathetic function in elderly population. Their primary outcome measure was post-prandial change in beat-to-beat heart rate variation, using Heart rate spectral analysis of a Holter monitor, and blood pressure in the two groups of participants. In young participants, the low-frequency HR power and the low to high frequency band ratio increased suggesting activation of sympathetic nervous system. The elderly group did not have such response after a meal. Furthermore, the elderly group had significant drop in
blood pressure after a meal indicating poor sympathetic activation.

Furthermore another study investigated the post meal sympathetic activation amongst patients with chronic ANS insufficiency presenting with idiopathic orthostatic hypotension, patients who had surgical sympathetectomy for uncontrolled hypertension, and one patient with pheochromocytoma on phenoxybenzamine, alpha adrenergic receptor blocker, against healthy controls (Robertson, Wade et al. 1981). After a mixed meal the mean arterial pressure dropped by 42 mmHg and 28 mmHg in sympathectomy group and a patient on phenoxybenzamine for pheochromocytoma respectively. The control group had mean increase of systolic blood pressure by 1 mmHg and diastolic pressure by 4 mmHg after a mixed meal. The study concluded that there was activation of sympathetic nervous system after a meal and it was blocked by surgical sympathectomy, and a sympatholytic agent.

Therefore, a meal can stimulate sympathetic outflow and is useful in examining the function of the latter.

**Role of ANS in suppressing ghrelin**

Autonomic nervous system has been widely accepted as a control mechanism for ghrelin. Lee et al. reported increased ghrelin levels in vagotomized rats (Lee, Wang et al. 2002). It must be noted that ghrelin was measured after 14 days post vagotomy and the finding could be due to chronic changes rather than immediate withdrawal of vagal input. In another animal study, when Vagus nerve was stimulated for both 30 and 90 minutes, and it lead to reduction in plasma ghrelin significantly (Murakami, Hayashida et al. 2002). The study did not assess the role of Vagus nerve in post- prandial suppression of ghrelin but indirectly suggested a role of Vagus nerve on ghrelin at resting state. In a human study authors used a modified sham feeding to induce vagal stimulation and assessed its effect on post- meal ghrelin suppression (Heath, Jones et al. 2004). Plasma ghrelin levels are responsive to short- and long-term nutrient fluctuation, rapidly decreasing with food consumption and increasing with food deprivation or weight loss. William et al. hypothesized a vagal contribution to both responses. Nutrient-related ghrelin suppression may be mediated by gastrointestinal load-related vagal afferent activity, or depend upon vagal efferent input to the foregut, where most ghrelin is produced. Similarly, the deprivation-induced ghrelin rise could require state-related vagal afferent or efferent activity. Hence, the authors examined the role of the vagus nerve in the regulation of plasma ghrelin by sampling blood from rats with subdiaphragmatic vagotomy and from
sham-operated controls over 48 h of food deprivation, and before and after gastric gavage of liquid diet. Vagotomy affected neither baseline ghrelin levels nor the suppression of ghrelin by a nutrient load. The food deprivation-induced elevation of plasma ghrelin levels, however, was completely prevented by subdiaphragmatic vagotony. In a separate experiment, the deprivation-related rise in plasma ghrelin was substantially reduced by atropine methyl nitrate treatment, indicating that the response to fasting is driven by increased vagal efferent tone. The dissociation between nutrient load- and deprivation-related ghrelin responses indicates that the regulation of circulating ghrelin levels involves separate mechanisms operating through anatomically distinct pathways (Williams, Grill et al. 2003).

Eight healthy individuals had both oral fat loads alone or modified sham feeding followed by oral fat load one hour after modified sham feeding. In the study, modified sham feeding was applied as a mean to stimulate vagus nerve. Plasma ghrelin level got lower after both protocols but greater suppression of ghrelin was noted in modified sham feeding followed by oral fat load. The authors reported vagal stimulation increased the pre-meal ghrelin level and suppressed the post-prandial ghrelin secretion. The effect of meal might also have effect on sympathetic outflow (Bray 1991, Ryan, Goldberger et al. 1992, Scott, Greenwood et al. 2013) which might have contributed to the ghrelin suppression. Another study did not find sham feeding, chewing and spitting, in health subjects suppressed plasma ghrelin level in healthy participants (Erdmann, Lippl et al. 2003). Ten healthy normal weight volunteers had modified sham feeding to stimulate cephalic vagal output and gastric dilation by 300 ml of guar solution. Modified sham feeding resulted in slight increase, and gastric dilation had no significant effect in ghrelin level. A recent study investigated the role of central melatoninergic tone on ghrelin suppression in human (Schubert and Makhlouf 1993). The study measured fasting and postprandial plasma total ghrelin in participants with melanocortin 4- receptor mutation (MC4RM), simple obesity and lean controls. The fasting total ghrelin level and AUC reduced in obese and lean group but ghrelin suppression was attenuated in MC4RM. Therefore central melanocortinergic tone modulates total ghrelin suppression.

**Hyperghrelininaemia in PWS**

In patients with PWS, the appetite is insatiable (Holland, Treasure et al. 1993) and it is generally assumed to be due to high and unregulated orexigenic peptide ghrelin (DelParigi, Tschop et al. 2002, Haqq, Farooqi et al. 2003, Butler, Bittel et al. 2004, Erdie-Lalena, Holm et al. 2006, Butler and Bittel 2007, Haqq, Grambow et al. 2008). However,
the direct causal link between high ghrelin level and increased appetite has not been proven to this stage. Only positive correlation was reported between blood ghrelin level and subjective rating of hunger in PWS (DelParigi, Tschop et al. 2002). DelParigi and Tschop et al. compared fasting plasma total ghrelin in PWS group and healthy controls (DelParigi, Tschop et al. 2002). The mean fasting plasma ghrelin in PWS group was significantly higher than the healthy control group. After adjusting for the percent body fat, hyperghrelinaemia in PWS group remained unchanged compared to the control group in the study. The authors also reported that there was positive correlation between plasma ghrelin level and subjective ratings of hunger. Haqq and Farooqi et al. measured fasting serum ghrelin in children with PWS and compared to four weight matched control groups, normal weight control group, obese control group, children with melanocortin-4 receptor mutation group, and leptin deficient control group (Haqq, Farooqi et al. 2003). The fasting ghrelin level in PWS group is significantly higher compared to obese, melanocortin-4 receptor mutation and leptin deficient control groups but was not significantly different to the normal weight controls.

Choe et al. reported that 16 children with PWS had 2-3 folds higher number of ghrelin expressing cells in the gastric fundus and 2-3 times higher ghrelin level compared to the 13 growth hormone deficient children, 10 obese controls and 19 lean controls (Choe, Song et al. 2005). In an another case-control study, forty children with PWS were compared to the eighty seven healthy non-obese controls (Feigerlova, Diene et al. 2008). The authors reported that children and adolescents with PWS had significantly higher plasma ghrelin level than the controls. Plasma ghrelin level was higher in very young PWS children than the controls and the study concluded it could play a role in development of early obesity in PWS.

**Suppressing ghrelin in PWS**

A meal or glucose load can suppress ghrelin in patients with PWS but not to a degree seen in the unaffected individuals (Haqq, Stadler et al. 2003, Paik, Choe et al. 2006, Gimenez-Palop, Gimenez-Perez et al. 2007). Gimenez-Palop et al. described suppression of plasma ghrelin in a case-control study that involved 7 PWS adults, who were not on growth hormone therapy; 7 obese control; and 7 lean controls (Gimenez-Palop, Gimenez-Perez et al. 2007). After a liquid meal, plasma ghrelin level in PWS got suppressed but it did in slower rate than that of the two control groups. 270 minutes after the meal the mean ghrelin AUC was higher than the obese control group. The authors suggested that the attenuation of ghrelin suppression after a meal might have relationship with hyperphagia in
PWS. Haqq et al. used a mixed meal to suppress ghrelin in PWS in an uncontrolled study (n=11) (Haqq, Stadler et al. 2003). Three participants had significant post-prandial suppression but the rest had slower suppression. Paik et al., however, demonstrated poor post-prandial suppression of acyl ghrelin after oral glucose load in a case-control study on 11 children with PWS and 10 simple obese controls (Paik, Choe et al. 2006). The mean post glucose load acyl ghrelin level in children with PWS was significantly higher than the obese controls. Fasting acyl ghrelin and change in acyl ghrelin after oral glucose load were found to be correlated to whole body insulin sensitivity in children with PWS. The current understanding of attenuated post-prandial suppression of ghrelin may be the explanation for poor satiation in PWS.

**Effect of growth hormone therapy on ghrelin suppression in PWS**

The current evidence of the effect of growth hormone therapy on ghrelin suppression in PWS is unclear. In a study, growth hormone therapy changed the ghrelin profile related to oral glucose load in PWS patients (Hauffa, Haase et al. 2007). The study included twenty-eight children with PWS, and investigated the status of plasma total and acyl ghrelin, before and after commencement of growth hormone therapy. Suppression of total ghrelin after glucose load was further reduced to a lower degree after the commencement of growth hormone therapy. However, the degree of post-glucose suppression of acyl ghrelin appeared similar after the prolonged use of growth hormone. In 2009 the same group published a letter that growth hormone therapy reduced the ghrelin status and it might pose as a confounder in studies on ghrelin status in PWS (Hauffa and Petersenn 2009). On the other hand, in one study, nine children with PWS who were younger than 3 years of age had two plasma ghrelin evaluations before and at median one year of growth hormone therapy (Feigerlova, Diene et al. 2008). In the study, the ghrelin levels were not significantly different before and after starting growth hormone therapy. In a randomized study, plasma ghrelin status in adult PWS participants did not change after 6 months and 12 months of growth hormone therapy (Hoybye, Barkeling et al. 2003). Growth hormone therapy has not been shown to influence plasma acyl ghrelin in current literature.
2.2: Clinical Questions and Hypothesis
1. Is there ANS dysfunction in PWS?
2. Does ANS dysfunction have a causal role in fasting acyl ghrelin and post-prandial suppression of acyl ghrelin?

Based on the available evidence, it is my hypothesis that in PWS, which is considered to be a hypothalamic disorder, there is ANS dysfunction and it in turn is the cause for poor ghrelin suppression in PWS.

2.3: Methods

Aims, study type and study populations

The aims of the study:
1. To compare ANS status at fasting and after a mixed meal in the PWS and control groups by examining three target sites of ANS, cardiovascular system for orthostatic change in pulse rate and blood pressure, stomach for gastrin production and chromaffin cells of adrenal medulla for catecholamine production.
2. To compare the fasting and post-prandial acyl ghrelin (AG) levels in PWS and control groups.
3. To correlate ANS function to the degree of AG suppression.

The study is a case-control study and there will be PWS group and age, gender and body mass index matched control group with simple obesity (Non-PWS).

Inclusion Criteria

PWS group consisted of 16 genetically diagnosed patients. The control group comprised of age, gender and BMI matched obese children without PWS.

This study was conceived and designed before the Commonwealth Government of Australia started to subsidized growth hormone to all children under 18 years with genetic diagnosis of PWS. At the time of the launch of the study, PWS participants were already on growth hormone therapy that posed as an unavoidable confounder of the study.

Exclusion Criteria

Any participants with history of having any type of Diabetes Mellitus, other hypothalamic pathologies such as cranial radiation, surgery or central neuronal disorders, use of Psychotrophics such as Risperidone, Selective Serotonin reuptake inhibitors, or use of psychoostimulants were excluded.
48 PWS patients who attended the PWS clinic at Mater Children’s Hospital were approached personally and by post. The first 16 agreed to participate were included in the study. There were 2 PWS participants of mixed Asian and Caucasian descent. The rest of PWS and the controls are Caucasians. One PWS patient with type 2 diabetes mellitus and one who was on psychotropic drug were excluded. Volunteers without features of and diagnosis of PWS were included as controls. Friends and siblings of PWS patients; and children who attend general endocrinology and obesity clinic were approached. Due to difficulty in recruiting controls, normal weight controls were not included in the study.

**Study design**

All participants were asked to have their regular food intake prior to the study and they fasted overnight for 10 hours during the night before mixed meal challenge test. The study was performed at Mater Children’s Hospital in the morning. The participants were asked to collect the first urine sample after waking for catecholamine measurement (Urine test). After arrival to the Mater Children’s hospital, the weight and height were measured to the nearest 0.1 kg and 0.1 cm. Then topical anaesthetic cream was applied at the site on the upper limb where an indwelling intravenous cannula was to be inserted. Then all participants had their body composition measured using Bodystat 1500®. Bodystat 1500® works by passing a safe, battery generated signal through the body and measures the bioelectrical impedance (BIA) at 50 kHz. The National Institutes of Health provided a consensus statement that BIA provides a reliable method of measuring body composition under most conditions (1996). BIA has been a simple and useful tool in both clinical and research settings (Kyle, Bosaeus et al. 2004, Verdich, Barbe et al. 2011). BIA was chosen to measure body composition because it is cheap and practical as a bed-side device. Dual energy X-ray absorptiometry, being superior to BIA, was not used because of its expense and radiation exposure.

The participants had sequential pulse, and blood pressure taken at supine and standing positions (Cardiovascular Test). A cannula was inserted for blood samplings and 15 minutes after cannulation a venous sample of blood was collected for fasting serum acyl ghrelin, catecholamine and gastrin (Blood test). Then a mixed meal, which was similar to a usual breakfast composing of 50 gm of Carbohydrate, 23.38 gm of protein and 13.38 gm of fat (Table 1 and 2) was given to the participants. After the meal, blood tests and cardiovascular tests were repeated at 10, 30, 60 and 120 minutes; and urine sample at 2 hours.
Table 1.
Contents of the mixed meal.

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muffin, English, Regular</td>
<td>70g</td>
</tr>
<tr>
<td>Egg, Whole, Raw</td>
<td>50g</td>
</tr>
<tr>
<td>Bacon, Middle, Grilled, Lean &amp; Fat</td>
<td>21g</td>
</tr>
<tr>
<td>Cheese, Cheddar, Reduced Fat (25% Reduction)</td>
<td>10g</td>
</tr>
<tr>
<td>Juice, Pineapple-orange, unsweetened</td>
<td>250ml</td>
</tr>
</tbody>
</table>

Table 2.
Components of the mixed meal.

**ANALYSES**

<table>
<thead>
<tr>
<th>Component</th>
<th>per meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>413.5g</td>
</tr>
<tr>
<td>Energy</td>
<td>1739.61KJ</td>
</tr>
<tr>
<td>Protein</td>
<td>23.38g</td>
</tr>
<tr>
<td>Total Fat</td>
<td>13.38g</td>
</tr>
<tr>
<td>- Saturated Fat</td>
<td>5.03g</td>
</tr>
<tr>
<td>- Poly-unsaturated Fat</td>
<td>1.34g</td>
</tr>
<tr>
<td>- Monounsaturated Fat</td>
<td>5.15g</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>213.18mg</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>50.02g</td>
</tr>
<tr>
<td>Sugars</td>
<td>25.59g</td>
</tr>
<tr>
<td>Starch</td>
<td>24.43g</td>
</tr>
<tr>
<td>Water</td>
<td>315.16g</td>
</tr>
<tr>
<td>Alcohol</td>
<td>0g</td>
</tr>
<tr>
<td>Dietary Fibre</td>
<td>2.36g</td>
</tr>
<tr>
<td>Thiamin</td>
<td>0.54mg</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.37mg</td>
</tr>
<tr>
<td>Niacin</td>
<td>4.68mg</td>
</tr>
<tr>
<td>Niacin Equivalents</td>
<td>10.04mg</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>71.3mg</td>
</tr>
<tr>
<td>Total Folate</td>
<td>57.60ug</td>
</tr>
<tr>
<td>Total Vitamin A Equivalents</td>
<td>120.80ug</td>
</tr>
<tr>
<td>Retinol</td>
<td>99.42ug</td>
</tr>
<tr>
<td>B-Carotene Equivalents</td>
<td>127.25ug</td>
</tr>
<tr>
<td>Sodium</td>
<td>858.45mg</td>
</tr>
<tr>
<td>Potassium</td>
<td>651.65mg</td>
</tr>
<tr>
<td>Magnesium</td>
<td>56.48mg</td>
</tr>
<tr>
<td>Calcium</td>
<td>203.57mg</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>350.28mg</td>
</tr>
<tr>
<td>Iron</td>
<td>2.36mg</td>
</tr>
<tr>
<td>Zinc</td>
<td>2.51mg</td>
</tr>
</tbody>
</table>

Percent change in pulse at 15 and 30 seconds of standing (%ΔP at 15 s and 30 s) was calculated from the difference in supine pulse rate to the pulse rates at 15 and 30 seconds after assumption of an upright posture. Similarly, the percentage of change in diastolic and systolic pressures (% Δ in DBP and SBP) were calculated from difference between supine
to standing blood pressures respectively at 15 and 30 seconds of standing from supine position (Hall, 1988). Furthermore, mean arterial pressure (MAP) and percent change in MAP were calculated at 15s and 30s after standing. Urinary catecholamine; ΔPR and ΔBP at 15 sec were measured to assess sympathetic nervous system. Plasma gastrin was for parasympathetic functions.

**Plasma acyl ghrelin**

Blood samples were collected in the presence of a protease inhibitor (1mg/ml, Pefabloc SC, Roche). Blood samples were spun immediately in a refrigerated bucket centrifuge for 15 mins at 3000 x g. Plasma was removed and one half was acidified with 1N HCl to a final concentration of 0.05N HCl in order to protect the acyl moiety. Plasma (acidified and non-acidified) was aliquoted into 100ul lots and frozen on dry ice and stored at -80°C. Acyl ghrelin was measured in plasma using a commercial ELISA (Human Active Ghrelin ELISA, Millipore, catalogue number EZGRA-88K), which displays no cross reactivity to des-octanoyl ghrelin. Values were interpolated from a sigmoidal 4- parameter logistic equation (GraphPad Prism 5.0) and intra-assay coefficient of variation was 5.9%.

**Plasma gastrin**

Gastrin is a hormone produced from the G cells at the base of gastric pyloric glands. It stimulates gastric acid secretion and mucosal cell growth (Chu and Schubert, 2012). Vagus nerve controls the gastrin secretion from the stomach.

Plasma gastrin was assayed using the MP Biomedicals double antibody competitive radioimmunoassay. The patient sample was incubated with anti-gastrin antibody (rabbit anti-human gastrin) and 125I-labelled gastrin which competed with gastrin in the patient sample for the antibody binding sites. After incubation, separation of bound from free gastrin was achieved by precipitation of the antibody-bound gastrin with a second antibody - goat anti-rabbit antibody plus a precipitation accelerator- followed by centrifugation and decantation of the unbound gastrin in the supernatant. The antibody-bound 125I- gastrin fraction was counted in a gamma counter and the counts for patients' samples were compared with those for a set of standards of known gastrin concentration. As the concentration of gastrin in the sample increased, the radioactivity present in the precipitate decreased.
### Table 3: Inter-assay variation of plasma gastrin assay. (2012)

<table>
<thead>
<tr>
<th>Level</th>
<th>Ng/L</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>49</td>
<td>10.0</td>
</tr>
<tr>
<td>L2</td>
<td>236</td>
<td>5.0</td>
</tr>
</tbody>
</table>

**Plasma catecholamine**

In human, catecholamine in circulation is mainly from the chromaffin cells of adrenal medulla. Adrenal catecholamine production results from two input pathways, sympathetic neurogenic input via splanchnic nerve to chromaffin cells (de Diego, Gandia et al. 2008) and local gap junctional coupling between chromaffin cells (Martin, Mathieu et al. 2001, Colomer, Desarmenien et al. 2009).

Metadrenaline, Normetadrenaline, and 3-Methoxy Tyramine in plasma were measured by Liquid Chromatography mass spectroscopy (LC-MSMS). Following the addition of a combined deuterated internal standard, samples at a neutral pH, were extracted from plasma by loading onto activated mixed mode cation exchange SPE columns. The columns were washed to remove interfering substances and the plasma metadrenalines eluted. The extract was dried down at 60°C, reconstituted in mobile phase and 20µL is injected onto a HILIC column. Multiple reactions monitoring (MRM) was then carried out for each individual analyte. Cycle time between samples is 5 minutes.
Table 4: Intra-assay precision of plasma metadrenaline and normetadrenaline
2 levels of Quality Controls were assayed 10 times in one run (2013).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Quality control</th>
<th>Mean</th>
<th>SD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metadrenaline</td>
<td>Chromsystems QC1</td>
<td>478</td>
<td>15</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>Chromsystems QC2</td>
<td>1765</td>
<td>20</td>
<td>3.1</td>
</tr>
<tr>
<td>Normetadrenaline</td>
<td>Chromsystems QC1</td>
<td>719</td>
<td>25</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>Chromsystems QC2</td>
<td>8394</td>
<td>306</td>
<td>3.6</td>
</tr>
</tbody>
</table>
Table 5: Inter-assay precision of plasma metadrenaline and normetadrenaline
2 levels Quality Controls were assayed over 20 runs (2013)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Quality Control</th>
<th>Mean</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metadrenaline</td>
<td>Chromsystem QC1</td>
<td>464</td>
<td>26</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>Chromsystem QC2</td>
<td>1827</td>
<td>74</td>
<td>4.0</td>
</tr>
<tr>
<td>Normetadrenaline</td>
<td>Chromsystem QC1</td>
<td>758</td>
<td>42</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>Chromsystem QC2</td>
<td>8556</td>
<td>376</td>
<td>4.4</td>
</tr>
</tbody>
</table>
**Urinary catecholamine**

Urinary-free catecholamines were extracted from urine and their concentration was determined by reverse phase isocratic High Performance Liquid Chromatography (HPLC) coupled with Electrochemical Detection (ECD). The extraction procedure involved two steps: cation exchange and alumina extraction. Urine was loaded onto disposable columns containing cation exchange resin; the resin bound catecholamines were then eluted with boric acid. Alumina was added to this eluate to extract the catecholamine, which were then eluted from the alumina by acetic acid. A small volume of this eluate was injected onto the isocratic HPLC system. A NovaPak C$_{18}$ reverse phase column separated the individual catecholamine peaks and the internal standard peak (IS). Peak resolution was improved by the addition of an ion-pairing reagent, 1-octane sulphonic acid, to the mobile phase. The catecholamine (CATS) were quantitated by ECD where they were oxidized to their corresponding anthraquinones (i.e. the oxidation of the phenolic group) at a fixed voltage potential and subsequently reduced back to their original state. The resulting flow of electrons was measured as current.
Table 6: Inter-assay precision of urinary catecholamine
BIORAD Lyphochek 1 and 2 urine QC’s were assayed over a 1-year period (2011)

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>CV%</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Noradrenaline</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1</td>
<td>256</td>
<td>13</td>
<td>5.0</td>
<td>70</td>
</tr>
<tr>
<td>L2</td>
<td>1203</td>
<td>69</td>
<td>5.7</td>
<td>70</td>
</tr>
<tr>
<td><strong>Adrenaline</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1</td>
<td>72</td>
<td>6</td>
<td>7.8</td>
<td>70</td>
</tr>
<tr>
<td>L2</td>
<td>454</td>
<td>30</td>
<td>6.5</td>
<td>70</td>
</tr>
<tr>
<td><strong>Dopamine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1</td>
<td>427</td>
<td>36</td>
<td>8.4</td>
<td>70</td>
</tr>
<tr>
<td>L2</td>
<td>3447</td>
<td>221</td>
<td>6.4</td>
<td>70</td>
</tr>
</tbody>
</table>
Autonomic nervous functions as measured by the orthostatic change in pulse rate and blood pressure at the fasting state and post-prandial stimulated states were compared between the PWS and control groups to examine the hypothesis. Fasting and post-prandial plasma gastrin, and plasma and urine catecholamine were also compared between PWS and control groups to complement cardiovascular data. Fasting and post-prandial plasma acyl ghrelin levels, and the rate of post-prandial reduction in plasma acyl ghrelin were also compared between the groups. Simple correlation was used to assess if there was a relation between ANS function and acyl ghrelin profile.

2.4: Statistics
The primary outcomes of the study were the level of ANS stimulation and suppression in serum ghrelxin level after a meal. There are no such data available in normal healthy population, thus the calculation of sample size using standard methods is not feasible. Nevertheless we know that if we have 16 children in each group (PWS and Controls) we will be able to detect a significant difference of 1 standard deviation in the primary outcome variable, if it exists. This would be clinically and biologically significant. This calculation is based upon the following:

In any calculation the sample size (n) can be determined by
\[ n = \frac{16}{f^2} \]
where \( f \) is defined as

\[ f = \frac{\text{biologically significant difference to detect}}{\text{standard deviation of the parameter of interest}} \]

Thus in the proposed study, if we power the sample size to detect 1 SD of difference. Hence \( f \) equals to 1 and \( n \) equals 16.

SPSS statistics version 22 for Mac (IBM Analytic Software) was used to perform statistical tests. Student t tests was used to compare the means of the cardiovascular data in which two tail p value of <0.05 to reject the null hypothesis. Biochemical data is not normally distributed and it is expressed as median and inter-quartile range. Mann- Whitney U test was performed to compare the PWS and control groups. To correlate the fasting plasma acyl ghrelin level, and the fasting percent change of pulse rate, Pearson’s correlation was used. Area under curves (AUC) for acyl ghrelin and gastrin were calculated using
trapezoid method and those were compared using Mann-Whitney U test as those were not normally distributed. AUC of analytes are described as median and 95% confidence interval.

2.5: Ethical approval
The study has been approved by the HREC of the University of Queensland (Reference No. 2010000367) and Mater Health Services (Ref No. 1654C).

2.6: Results
The characteristics of the participants are described in the table 7. The PWS group comprised of 16 genetically confirmed individuals (nine females and seven males) and the control group had 16 volunteers (six females and 10 males). Apart from three of the controls, no other participants in both PWS and controls were in puberty. Three of the PWS participants were on stable thyroxine therapy for central hypothyroidism. The median age of the PWS cohort was 9.32 years (Inter quartile range 5.29) and due to difficulty in recruiting matched controls, older volunteers were accepted as controls. The median age for control group was 12.16 years (inter quartile range 6.12, p = 0.078). The PWS participants are shorter compared to the controls. The median height Z score for PWS group was -0.39 (IQR 1.45) while that of the controls was 1.03 (IQR 1.61, p= 0.049). The weight Z score in PWS group was 1.05 (IQR 1.62) and for the controls, 1.26 (IQR 1.32). The difference between groups did not reach statistical significance (p value of 0.54). The median BMI Z score in PWS group was 1.50 (IQR 1.39) and the control group was 1.10 (IQR 1.11, p = 0.423). The lean mass in significantly lower in PWS group compared to the control group (26.00%, IQR 12.48 and 44.84%, IQR 20.85 respectively, p= 0.013). Similarly, waist and height ratio as an indicator of adiposity, trends to be higher in PWS cohort. The median waist and height ratio in PWS group was 0.55 (IQR 0.27) and that of the control group was 0.5 (IQR 0.1, p = 0.055). PWS participants had been on stable growth hormone therapy and the mean plasma insulin-like growth factor 1 (IGF1) was within normal range in females but slightly higher than normal upper limit in males. The mean (SD) plasma IGF1 level for the whole group was 48.7 (28.2) nmol/l. The mean (SD) IGF1 level for male PWS participants was 47.6 (29.5) nmol/l (reference range: 34.9-45.2 nmol/l) and that of female participants was 47.6 (28.7) nmol/l (reference range: 33.2- 54.4).
Table 7:
Comparison of characteristics of the PWS and control groups

<table>
<thead>
<tr>
<th></th>
<th>PWS</th>
<th>Control</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Yr)</td>
<td>9.32 (5.29)</td>
<td>12.16 (6.12)</td>
<td>0.078</td>
</tr>
<tr>
<td>Median (IQR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height Z score</td>
<td>-0.39 (1.45)</td>
<td>1.03 (1.61)</td>
<td>0.049</td>
</tr>
<tr>
<td>Median (IQR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight Z score</td>
<td>1.05 (1.62)</td>
<td>1.26 (1.32)</td>
<td>0.545</td>
</tr>
<tr>
<td>Median (IQR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI Z score</td>
<td>1.50 (1.39)</td>
<td>1.10 (1.11)</td>
<td>0.423</td>
</tr>
<tr>
<td>Median (IQR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waist to height ratio</td>
<td>0.55 (0.27)</td>
<td>0.50 (0.1)</td>
<td>0.055</td>
</tr>
<tr>
<td>Median (IQR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lean mass %</td>
<td>26.00 (12.48)</td>
<td>44.84 (20.85)</td>
<td>0.013</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.6.1: Cardiovascular changes
Orthostatic compensatory autonomic response was poor in PWS participants. In PWS group, the lying pulse rate was higher than control group at both fasting post-prandial time points. But both of the pulse rates at 15 and 30 seconds of standing are lower than control group although the difference does not reach statistical significance (Figure 5). In other words, the mean pulse rate was higher in PWS participants than the controls but physiological orthostatic rise in pulse rate was lower in PWS participants than the controls.
Figure 5: Comparison of pulse rates while recumbent and standing at 15s (A) and 30s (B). Pulse rate is higher at recumbent position but orthostatic rise is attenuated in PWS group.

(A)

(B)

At the fasting state, the mean percent change in pulse rate at 30 seconds after standing from the recumbent position in PWS group is significantly lower than the control group. At 15 second, PWS group’s mean percent change in pulse rate (SD) was 10.2 % (± 3.66) and the control group’s was 23.22 % (± 7.54, p= 0.111). At 30 seconds, it was similarly lower
in PWS group than the control group (7.49 ± 3.42% and 28.9 ±5.06 respectively, p= 0.001).

After a mixed meal, it remained significantly lower at 10, 60 and 120 minutes in PWS group than the controls. At 10 minutes after a mixed meal, the mean percent change in pulse rate at 15 second after standing in PWS group decreased to 4.43% (± 3.56), and in the control group, it increased to 28.5% (± 6.68, p = 0.002). After 30 seconds of standing, it also decreased to 4.98 ± 5.60% in PWS group, and it increased to 32.04 ±7.29% in the control group (p = 0.006).

The percent change in pulse rate continued to rise in controls but that of the PWS subjects continued to fall at 60 minutes after a mixed meal. At 15 second of standing, the percent change of pulse rate was 4.43 ± 4.27% for PWS cohort and 35.04 ± 11.6% in controls (p= 0.013). At 30 seconds of standing, it was 4.55 ± 4.44% in PWS and 40.43 ± 10.17% in controls (p= 0.002).

The rise in pulse rate after standing was sustained in the controls 120 minutes after a mixed meal, but not in PWS group. The percent change of pulse rate was 4.70 ± 3.44% and 4.45 ± 4.78% in PWS, and 24.30 ±3.41% and 30.12 ± 4.13% in controls at 15 seconds (p= 0.0005) and 30 seconds (p= 0.0006) after standing respectively. (Table 8, Figure 6 and 7)
Table 8:
Comparison of change in orthostatic pulse rate in PWS and control groups

<table>
<thead>
<tr>
<th></th>
<th>PWS</th>
<th>Control</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fasting</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% change at 15s</td>
<td>10.2</td>
<td>23.22</td>
<td>0.111</td>
</tr>
<tr>
<td></td>
<td>(3.66)</td>
<td>(7.54)</td>
<td></td>
</tr>
<tr>
<td>% change at 30s</td>
<td>7.49</td>
<td>28.9</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>(3.42)</td>
<td>(5.06)</td>
<td></td>
</tr>
<tr>
<td><strong>10 minutes Postprandial</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% change at 15s</td>
<td>4.43</td>
<td>28.5</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>(3.56)</td>
<td>(6.68)</td>
<td></td>
</tr>
<tr>
<td>% change at 30s</td>
<td>4.98</td>
<td>32.04</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>(5.60)</td>
<td>(7.29)</td>
<td></td>
</tr>
<tr>
<td><strong>60 minutes Postprandial</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% change at 15s</td>
<td>4.43</td>
<td>35.04</td>
<td>0.013</td>
</tr>
<tr>
<td></td>
<td>(4.2)</td>
<td>(11.6)</td>
<td></td>
</tr>
<tr>
<td>% change at 30s</td>
<td>4.55</td>
<td>40.43</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>(4.44)</td>
<td>(10.17)</td>
<td></td>
</tr>
<tr>
<td><strong>120 minutes Postprandial</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% change at 30s</td>
<td>4.70</td>
<td>24.30</td>
<td>0.0005</td>
</tr>
<tr>
<td></td>
<td>(3.44)</td>
<td>(3.41)</td>
<td></td>
</tr>
<tr>
<td>% change at 30s</td>
<td>4.45</td>
<td>30.12</td>
<td>0.0006</td>
</tr>
<tr>
<td></td>
<td>(4.78)</td>
<td>(4.13)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 6:
The comparison of trends of % change in PR at 15 s after standing from recumbent position. DPR 15s means percent change of pulse rate at 15 seconds of standing from that of recumbent position.
* p = 0.006, $p < 0.02, # p = 0.0005
Figure 7:
The comparison of trends of % change in PR at 30 s after standing from recumbent position. DPR 30s means percent change of pulse rate at 30 seconds of standing from that of recumbent position.

**p= 0.001, * p= 0.006, $p<0.02, #p= 0.0005

With regards to change in systolic blood pressure (Table 9, Figure 8 & 9), the percent change in systolic pressure, after standing for 15 second from recumbent position, increased significantly 120 minutes after a mixed meal in the controls but it decreased in PWS group. It was 4.02 ± 4.08%, 1.60 ± 2.39%, 3.85 ±2.80% and -1.90 ±1.59% in PWS group at fasting, 10 minutes, 60 minutes and 120 minutes after a mixed meal. In the controls, it increased from 0.97 ± 3.32% at fasting state to 3.20 ± 2.12%, 5.10 ± 2.60% and 4.39 ±1.91% at 10 minutes, 60 minutes and 120 minutes after meal (difference with PWS, p values = 0.58, 0.64, 0.75, 0.017 respectively). The percent changes in systolic blood pressure at 30 seconds after standing was 2.35 ± 2.48% at fasting in the healthy controls but was -6.03 ± 2.30% in the PWS group (p= 0.02). The difference at 10 and 60 minutes post meal did not reach statistical significance (0.20 0.20 ± 2.31, 1.81 ± 2.98% for the control and -0.28 ± 2.83%, -2.37 ± 1.33% for PWS group). But at 120 minutes post meal, the percent changes in systolic blood pressure increased significantly (3.02 ± 1.43%) but that of PWS group decreased further (-3.55 ± 1.89%, p= 0.013).
Figure 8:
The comparison of trends of % change in systolic blood pressure at 15 s after standing from recumbent position. DSBP 15s means percent change of systolic blood pressure at 15 seconds of standing from that of recumbent position.
*p= 0.017

![Graph](image1)

Figure 9:
The comparison of trends of % change in systolic blood pressure at 30 s after standing from recumbent position. DSBP 30s means percent change of systolic blood pressure at 30 seconds of standing from that of recumbent position.
#p= 0.02, @p=0.013

![Graph](image2)
The difference in changes in diastolic blood pressure to standing did not reach statistical significance. In PWS cohort, the percent change in diastolic pressure at 15 seconds of standing was 7.89 ± 4.44%, 10.45 ± 5.58%, 7.67 ± 4.47% and 1.54 ± 4.15% at fasting, 10, 60 and 120 minutes. For the controls, it was 10.24 ± 2.82%, 9.84 ± 7.19%, 13.34 ± 5.05%, and 8.85 ± 3.49% respectively (difference to PWS group, p= 0.69, 0.95, 0.41, 0.20).

Although it was not statistically significant, percent change in diastolic blood pressure at 30 second increased at 10 minutes post meal but declined from then on in both of the groups. The magnitude of change in PWS is lower than the controls. After 30 seconds of standing, the percent change of diastolic blood pressure was -0.49 ± 2.87%, 5.94 ± 4.26%, 2.66 ± 2.61% and -1.27 ± 3.44% at fasting, 10, 60 and 120 minutes in PWS group. Whereas for the controls, it was 5.41 ± 4.85%, 13.16 ± 5.52%, 10.29 ± 3.06% and 4.62 ± 5.08% at fasting, 10 minutes, 60 minutes and 120 minutes after a meal (p value for difference to PWS group: 0.28, 0.30, 0.07, 0.33).
Table 9: Comparison of orthostatic change in systolic blood pressure in PWS and control groups

<table>
<thead>
<tr>
<th></th>
<th>PWS</th>
<th>Control</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fasting</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% change at 15s</td>
<td>4.02 (4.08)</td>
<td>0.97 (3.32)</td>
<td>0.58</td>
</tr>
<tr>
<td>% change at 30s</td>
<td>-6.03 (2.30)</td>
<td>2.35 (2.48)</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>10 minutes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postprandial</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% change at 15s</td>
<td>1.60 (2.39)</td>
<td>3.20 (2.12)</td>
<td>0.64</td>
</tr>
<tr>
<td>% change at 30s</td>
<td>-0.28 (2.83)</td>
<td>0.20 (2.31)</td>
<td>0.90</td>
</tr>
<tr>
<td><strong>60 minutes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postprandial</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% change at 15s</td>
<td>3.85 (2.80)</td>
<td>5.10 (2.60)</td>
<td>0.75</td>
</tr>
<tr>
<td>% change at 30s</td>
<td>-2.37 (1.33)</td>
<td>1.81 (2.98)</td>
<td>0.175</td>
</tr>
<tr>
<td><strong>120 minutes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postprandial</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% change at 15s</td>
<td>-1.90 (1.59)</td>
<td>4.39 (1.91)</td>
<td>0.017</td>
</tr>
<tr>
<td>% change at 30s</td>
<td>-3.55 (1.89)</td>
<td>3.02 (1.43)</td>
<td>0.013</td>
</tr>
</tbody>
</table>
Table 10: Comparison of change in diastolic blood pressure in PWS and control groups

<table>
<thead>
<tr>
<th></th>
<th>PWS</th>
<th>Control</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fasting</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% change at 15s</td>
<td>7.89 (4.44)</td>
<td>10.24 (2.82)</td>
<td>0.69</td>
</tr>
<tr>
<td>% change at 30s</td>
<td>-0.49 (2.87)</td>
<td>5.41 (4.85)</td>
<td>0.28</td>
</tr>
<tr>
<td><strong>10 minutes Postprandial</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% change at 15s</td>
<td>10.45 (5.58)</td>
<td>9.84 (7.19)</td>
<td>0.95</td>
</tr>
<tr>
<td>% change at 30s</td>
<td>5.94 (4.26)</td>
<td>13.16 (5.52)</td>
<td>0.30</td>
</tr>
<tr>
<td><strong>60 minutes Postprandial</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% change at 15s</td>
<td>7.67 (4.47)</td>
<td>13.34 (5.05)</td>
<td>0.41</td>
</tr>
<tr>
<td>% change at 30s</td>
<td>2.66 (2.61)</td>
<td>10.29 (3.06)</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>120 minutes Postprandial</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% change at 15s</td>
<td>1.54 (4.15)</td>
<td>8.85 (3.49)</td>
<td>0.20</td>
</tr>
<tr>
<td>% change at 30s</td>
<td>-1.27 (3.44)</td>
<td>4.62 (5.08)</td>
<td>0.33</td>
</tr>
</tbody>
</table>
Figure 10:
The comparison of trends of % change in diastolic blood pressure at 15 s after standing from recumbent position. DDBP 15s means percent change of diastolic blood pressure at 15 second of standing from that of recumbent position.  
p= NS

Figure 11:
The comparison of trends of % change in diastolic blood pressure at 30 s after standing from recumbent position. DDBP 30s means percent change of diastolic blood pressure at 30 second of standing from that of recumbent position.  
p= NS
Figure 12:
Comparison of change in mean arterial pressure at 15 and 30 seconds of standing (* p=0.045). %DMAP means percent change of mean arterial pressure at standing position (15 and 30 seconds) from mean arterial pressure at recumbent position.

Percent change of mean arterial pressure (MAP) at 15 second of standing was similar in both groups at fasting and 10 minutes post meal (Figure 12). The mean percent changes in MAP were 5.62 (SD13.67) % and 5.9 (SD 10.49) % at fasting; and 6.12 (SD 14.35) % and 6.27 (SD 13.19) % at 10 minutes post-meal in PWS and control groups respectively (p- NS). At 60 minutes post meal, PWS group’s percent change in MAP was 5.77 (SD 13.28) % and for the controls, 9.33 (SD 12.19) % (p- NS). However at 120 minutes post meal, there was further decrease in percent change of MAP in PWS group [-0.24(SD 10.62) % in PWS versus 6.69 (SD 8.16) %, p= 0.05].

At 30 seconds of standing, the difference in percent change of MAP in both groups did not reach statistical significance at fasting and 10 minutes post meal time points [-3.31 (SD 9.1) % and 2.82 (SD 11.38) % in PWS group; and 4.02 (SD 11.46) % and 6.58 (SD 11.04) % in the control group respectively] (Figure 12). But at 60 and 120 minutes post mixed meal, the percent change in MAP was significantly lower in PWS group [ 0.39 (SD 7.39)%]
and -2.34 (SD 10.23)% in PWS group; and 6.19 (8.31)% and 3.68 (SD 9.51)% in control group respectively, p=0.045 and 0.05] (Table 10).
Table 11:
Comparison of percent change in mean arterial pressure [mean (SD) %]

*P=0.045

**p= 0.05

<table>
<thead>
<tr>
<th></th>
<th>15 second</th>
<th>30 second</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PWS</td>
<td>Control</td>
</tr>
<tr>
<td>0 min</td>
<td>5.62 (13.67)</td>
<td>5.90 (10.49)</td>
</tr>
<tr>
<td>10 min</td>
<td>6.12 (14.35)</td>
<td>6.27 (13.19)</td>
</tr>
<tr>
<td>60 min</td>
<td>5.77 (13.28)</td>
<td>9.33 (12.19)</td>
</tr>
<tr>
<td>120 min</td>
<td>-0.24 (10.62)</td>
<td>6.69** (8.16)</td>
</tr>
</tbody>
</table>
Our findings suggest that there is poor sympathetic nervous reflex response to standing in PWS group. Immediately after standing from recumbent position, the sympathetic nervous system gets stimulated to increase adrenergic tone and maintain the cardiac output to maintain adequate blood flow to all organs particularly to the brain (Borst et al., 1984; Borst et al., 1982). It is represented in control group by higher percent change of pulse rate and arterial blood pressures, that is higher pulse rate and blood pressure at standing than recumbent position. But in PWS group the homeostatic rise in pulse rate failed to occur after 30 seconds of standing at fasting state and all post-prandial time points. Orthostatic change in systolic blood pressure at 120 minutes post meal was also significantly lower in the PWS group than the control group who displayed physiological rise in systolic blood pressure. Similarly, orthostatic rise in mean arterial pressure after 30 seconds of standing was lower in PWS group at 60 and 120 minutes post mixed meal. Although statistically insignificant, there was a trend towards lower orthostatic change in mean arterial pressure after 15 seconds of upright position at 120 minutes post meal.
**2.6.2: Biochemical changes**

**2.6.2.1: Plasma gastrin**

Figure 13:
Comparison of plasma gastrin profiles in PWS and control groups

At the baseline, plasma gastrin level was not different in both groups. Its level in both groups rose after the mixed meal but it rose even further in PWS compared to the control participants at 120 minutes after meal.

The median fasting plasma gastrin levels were 50.00 ng/L (IQR 20) in PWS group and 39.50 ng/L (IQR 25.75) in the control group (p 0.626). The control’s plasma gastrin level rose slightly at 30 minutes after the mixed meal to 88.00 ng/L (IQR 72.00) but that of PWS was higher at 170.50 ng/L (IQR 266.25). The difference did not reach statistical significance (p by Mann-Whitney U test = 0.37). At 60 minutes post-prandial time point, the median gastrin levels were 90.00 ng/L (IQR 53.00) and 100.00 ng/L (IQR 62.00) for PWS and controls respectively (p by Mann Whitney U test = 0.799). At 120 minutes after the mixed meal, the median plasma gastrin levels were 124.00 ng/L (IQR 98.50) and 69.00 ng/L (IQR 43.00) in PWS and controls respectively. The p value by Mann-Whitney U test is significant at 0.036 (Figure 13).

Furthermore, plasma gastrin area under curve (AUC) was significantly higher in PWS group. AUC$_{0-120}$ min for PWS and control groups were 13753.12 ng.min/L (95% CI 10196.83 to 17309.42) and 9378.46 ng.min/L (95% CI 6865.26 to 11891.66) (p 0.045) (Table 11).
Table 12:
Fasting and post-prandial plasma gastrin levels (ng/L) and gastrin AUC \(0\text{--}120\) min (ng.min/L) in PWS and control groups. The gastrin levels are expressed in median (inter-quartile range) and gastrin AUC \(0\text{--}120\) min with 95% confidence interval.

<table>
<thead>
<tr>
<th></th>
<th>PWS</th>
<th>Control</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting</td>
<td>50.00</td>
<td>39.50</td>
<td>0.626</td>
</tr>
<tr>
<td></td>
<td>(20.00)</td>
<td>(25.75)</td>
<td></td>
</tr>
<tr>
<td>30 min postprandial</td>
<td>170.50</td>
<td>88.00</td>
<td>0.370</td>
</tr>
<tr>
<td></td>
<td>(266.25)</td>
<td>(72.00)</td>
<td></td>
</tr>
<tr>
<td>60 min postprandial</td>
<td>90.00</td>
<td>100.00</td>
<td>0.799</td>
</tr>
<tr>
<td></td>
<td>(53.00)</td>
<td>(62.00)</td>
<td></td>
</tr>
<tr>
<td>120 min postprandial</td>
<td>124.00</td>
<td>69.00</td>
<td>0.036</td>
</tr>
<tr>
<td></td>
<td>(98.50)</td>
<td>(43.00)</td>
<td></td>
</tr>
<tr>
<td>(P) gastrin AUC (0\text{--}120) min</td>
<td>13753.12</td>
<td>9378.46</td>
<td>0.045</td>
</tr>
<tr>
<td></td>
<td>(10196.83 to 17309.42)</td>
<td>(6865.26 to 11891.60)</td>
<td></td>
</tr>
</tbody>
</table>

2.6.2.2: Catecholamine's
The mean plasma normetadrenaline levels were 287.1 ± 18.55 pmol/L, 319.8 ± 22.53 pmol/L, 298.2 ± 33.25 pmol/L, and 330.6 ± 29.1 pmol/L at fasting, 30 minutes, 60 minutes and 120 minutes after meal in PWS group. The levels were similar in the controls, 283.4 ± 31.96 pmol/L, 288.6 ± 30.1 pmol/L, 269.7 ± 29.28 pmol/L and 279.8 ± 26.22 pmol/L at fasting, 30 minutes, 60 minutes and 120 minutes post meal (p value for difference between PWS group: not significant at all-time points). In similar manner, the mean plasma metadrenaline levels were not significantly different between the two groups. In PWS group, it was 190.2 ± 18.94 pmol/L at the fasting state, 174.9 ± 16.8 pmol/L at 30 minutes post meal, 163.5 ± 13.28 pmol/L at 60 minutes post meal, and 165.4 ± 16.28 pmol/L at 120 post meal whereas it was 191.5 ± 23.17 pmol/L at fasting state, 167.6 ± 24.35 pmol/L at 30 minutes post meal, 144.2 ± 16.90 pmol/L at 60 minutes post meal, and 181.5 ± 27.56 pmol/L at 120 minutes post meal (difference with PWS: p = not significant at all-time points).
Figure 14:
Plasma normetadrenaline (A) and metadrenaline (B) profiles in PWS and control groups

(A)

P Normetadrenaline profile

(B)

P Metadenaline profile

Control
PWS
Table 13:
Fasting and post-prandial plasma normetadrenaline and metadrenaline levels in PWS and control groups [mean and SEM]. The difference of both analytes at all time point do not reach statistical significance.

<table>
<thead>
<tr>
<th>Time Point</th>
<th>(P) normetadrenaline (pmol/L)</th>
<th>(P) metadrenaline (pmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PWS</td>
<td>Control</td>
</tr>
<tr>
<td>Fasting</td>
<td>287.10(18.53)</td>
<td>283.40(31.96)</td>
</tr>
<tr>
<td>30 min postprandial</td>
<td>319.80(22.53)</td>
<td>288.60(30.10)</td>
</tr>
<tr>
<td>60 min postprandial</td>
<td>298.20(33.25)</td>
<td>269.70(29.28)</td>
</tr>
<tr>
<td>120 min postprandial</td>
<td>330.60(29.10)</td>
<td>27.98(26.22)</td>
</tr>
</tbody>
</table>

Urinary catecholamines excretion however was higher in PWS than controls at both fasting and at 2 hours post meal. Mean urinary Vanillylmandelic acid and creatinine ratio was 2.45 ± 0.20 umol/ mol and 1.87 ± 0.17 umol/ mol at fasting state in PWS and controls respectively (p for the difference= 0.037). It was 2.68 ± 0.19 umol/ mol and 1.86 ± 0.19 umol/ mol at 2 hours post- prandial time point in PWS and controls respectively (p for the difference= 0.006). At fasting, the mean urinary noradrenaline and creatinine ratio was 24.15 ± 2.67 umol/ mol in PWS group, but in the controls, it was significantly lower at 15.00 ± 1.63 umol/ mol (p for the difference = 0.006). It remained different at two hours post meal in two groups. Mean noradrenaline and creatinine ratio in urine was 39.57 ± 4.05 umol/ mol in PWS group and 24.00 ± 4.84 umol/ mol in the controls (p = 0.020). Furthermore, mean urinary adrenaline and creatinine ratio while fasting in PWS cohort was 2.62 ± 0.51 umol/ mol whereas it was 0.97 ± 0.16 umol/ mol in control cohort (p = 0.003). At two hours post meal, it continued to rise significantly in PWS participants. It was 8.63 ± 1.90 umol/ mol in PWS group but 4.53 ± 0.93 umol/ mol in control group (p = 0.088).

In PWS group, urinary excretion of dopamine, normetadrenaline, metadrenaline and 3 methoxy tyrosine expressed as the analytes and creatinine ratios were 325.5 ± 38.57 mmol/ mol, 0.12 ± 0.01 mmol/ mol, 0.07 ± 0.01 mmol/ mol and 0.10 ± 0.01 mmol/ mol at
fasting respectively. Those of the control group at baseline were similar at 252.9 ± 23.34 mmol/ mol, 0.11 ± 0.01 mmol/ mol, 0.07 ± 0.01 mmol/ mol, and 0.08 ± 0.01 mmol/ mol respectively (p for difference between groups = 0.11, 0.49, 0.73 and 0.18). However, at 2 hours after a mixed meal, urinary dopamine, normetadrenaline, metadrenaline and 3 methoxy tyrosine excretion in PWS were higher than the controls. 2 hours post meal mean urinary dopamine and creatinine ratio was 296.0 ± 28.14 mmol/ mol in PWS and 227.0 ± 26.16 mmol/ mol in controls (p = 0.085), mean urinary normetadrenaline and creatinine ratio was 0.15 ± 0.01 mmol/ mol in PWS and 0.11 ± 0.01 mmol/ mol in controls (p = 0.016), mean urinary metadrenaline and creatinine ratio was 0.10 ± 0.02 mmol/ mol in PWS and 0.07 ± 0.01 mmol/ mol in controls (p = 0.20), and mean urinary 3 methoxy tyrosine and creatinine ratio was 0.12 ± 0.01 mmol/ mol in PWS and 0.07 ± 0.01 mmol/ mol in controls (p = 0.026). Early morning urine is the urine produced overnight which is stored in the urinary bladder and catecholamine level in it reflects the level of adrenergic activity during sleep. Early morning urine samples of PWS participants showed higher vanillylmandelic acid, adrenaline and noradrenaline than the controls (p<0.05). Therefore, it reflects higher catecholamine production during sleep and is unlikely that PWS participants are more acutely stressed than the controls.
Table 14:
Fasting and post-prandial urinary catecholamines and creatinine ratios in PWS and control groups (mean and SEM)
* p< 0.05
**p<0.01

<table>
<thead>
<tr>
<th></th>
<th>PWS</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fasting 120 min postprandial</td>
<td>Fasting 120 minutes postprandial</td>
</tr>
<tr>
<td>Vanillylmandelic acid</td>
<td>2.45* (0.20)</td>
<td>2.68** (0.19)</td>
</tr>
<tr>
<td>(umol/mol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Noradrenaline (umol/mol)</td>
<td>24.15** (2.67)</td>
<td>39.57* (4.05)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15.00** (1.63)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24.00* (4.84)</td>
</tr>
<tr>
<td>Adrenaline (umol/mol)</td>
<td>2.62** (0.51)</td>
<td>8.63 (1.90)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.97** (0.16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.53 (0.93)</td>
</tr>
<tr>
<td>Dopamine (mmol/mol)</td>
<td>325.50 (38.57)</td>
<td>296.00 (28.14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>252.90 (23.34)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>227.00 (26.16)</td>
</tr>
<tr>
<td>Normetadrenaline (mmol/mol)</td>
<td>0.12 (0.01)</td>
<td>0.15* (0.01)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.11 (0.01)</td>
</tr>
<tr>
<td>Metadrenaline (mmol/mol)</td>
<td>0.07 (0.01)</td>
<td>0.10 (0.02)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.07 (0.01)</td>
</tr>
<tr>
<td>3 methoxy tyrosine (mmol/mol)</td>
<td>0.10 (0.01)</td>
<td>0.12* (0.01)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.08 (0.01)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.07* (0.01)</td>
</tr>
</tbody>
</table>
Figure 15:
Comparison of urinary catecholamines and creatinine ratios in the PWS and control groups

![U VMA profile graph]

*p= 0.035
*p= 0.006
U Metadrenaline profile

U 3mtyr profile

* p = 0.026

U Normetadrenaline profile

* p = 0.015
2.6.2.3: Plasma acyl ghrelin

The mean fasting plasma acyl ghrelin was higher in PWS group than the controls but it decreased to similar levels at 60 minutes and 120 minutes after a mixed meal. The mean plasma acyl ghrelin levels were 764.2 ± 67.1 pg/ml, 631.2 ± 54.2 pg/ml, 490.1 ± 53.0 pg/ml, 414.3 ± 43.9 pg/ml, 304.3 ± 28.0 pg/ml, and 283.0 ± 28.9 pg/ml at fasting, 10 minutes, 20 minutes, 30 minutes, 60 minutes and 120 minutes after a mixed meal in PWS group. In the control group the mean plasma acyl ghrelin levels were 517.2 ± 67.3 pg/ml, 473.4 ± 67.4 pg/ml, 381.2 ± 56.1 pg/ml, 313.8 ± 46.4 pg/ml, 283.9 ± 38.2 pg/ml, and 279.6 ± 38.2 pg/ml at fasting, 10 minutes, 20 minutes, 30 minutes, 60 minutes and 120 minutes post meal (p values for the difference with PWS group= 0.021, 0.290, 0.647, 0.245, 0.697, and 0.689).

Table 15:
The mean and SEM of plasma acyl ghrelin levels (pg/ml) in PWS and control groups at fasting, 10 minutes, 20 minutes, 30 minutes, 60 minutes and 120 minutes after a mixed meal. (ns- Not significant)

<table>
<thead>
<tr>
<th></th>
<th>PWS</th>
<th>Control</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting</td>
<td>764.2 (67.1)</td>
<td>517.2 (67.3)</td>
<td>0.021</td>
</tr>
<tr>
<td>10 min</td>
<td>631.2 (54.2)</td>
<td>473.4 (67.4)</td>
<td>ns</td>
</tr>
<tr>
<td>20 min</td>
<td>490.1 (53.0)</td>
<td>381.2 (56.1)</td>
<td>ns</td>
</tr>
<tr>
<td>30 min</td>
<td>414.3 (43.9)</td>
<td>313.8 (46.4)</td>
<td>ns</td>
</tr>
<tr>
<td>60 min</td>
<td>304.3 (28.0)</td>
<td>283.9 (38.2)</td>
<td>ns</td>
</tr>
<tr>
<td>120 min</td>
<td>283.0 (28.9)</td>
<td>279.6 (38.2)</td>
<td>ns</td>
</tr>
</tbody>
</table>
The acyl ghrelin area under curve is higher in PWS group during the early post-prandial period. The median (IQR) acyl ghrelin AG AUC$_{0-10\text{min}}$ for PWS group was 6771.33 pg.min/ml (3833.32) and for controls, 4888.33 pg.min/ml (3429.74) (p value by Mann-Whitney U test 0.035). The fasting acyl ghrelin level in PWS cohort is negatively correlated to percent change in pulse rate at 30 seconds after assuming standing position from lying at fasting state (r value -0.518, p 0.04).

The rate of fall of acyl ghrelin was faster in PWS. The median (IQR) rate of decrease in plasma level of acyl ghrelin for PWS was - 3.47 (2.88) and controls, - 1.65 (2.38) pg/ml/min (p Mann Whitney U 0.006).

2.7: Discussion
The attenuated percent change in pulse rate after stimulation by a mixed meal suggests poorer sympathetic nervous function in PWS. The rise of systolic blood pressure in PWS group was also lower than the controls at 120 minutes after a mixed meal. Similarly, the mean arterial pressure did not rise during 15 and 30 seconds of orthostatic stimuli at 120 minutes; 60 and 120 minutes after a mixed meal respectively.

The neuronal component of sympathetic adrenergic action is believed to be responsible for the reflex homeostatic mechanism to maintain normal cardiac output and blood pressure. Hill et al reported the effect of gravity on circulation (Hill 1895, Hill and Barnard).
The authors studied the effects of gravity, anaesthetics, dividing vagi, dividing the spinal cord, dividing the splanchnics, asphyxia, and curare in intracranial and arterial pressures in animals. The animals were put on a tilting board with their legs strapped in extended position. When placed in the vertical feet-down position, the pressures dropped in the experimental animals. On restoring to the horizontal position, the pressures got restored to the original levels. After dividing spinal cord at 3rd to 6th dorsal vertebra, the orthostatic fall in the pressures were more pronounced. Division of splanchnic nerve produced similar drop in the pressures. Hill concluded that gravity was a cardinal hydrostatic force in conditions such as syncope and shock, and that the splanchnic vaso-motor compensatory mechanism was important. Standing up reduces venous return to the heart by pooling blood at the dependent parts of the body. The afferent signals carried by Vagus nerve to the baro-receptors in the brain. The efferent adrenergic neurons increase the heart rate, cardiac output, and vascular resistance (Andersson, Cabero et al. 1996).

In a human study (Kelbaek, Munck et al. 1989), twenty three healthy subjects without past history of syncope underwent tilt table test. Transient fall of blood pressure was noted during 70-degree upright tilt. There was negative association between blood pressure and calf blood flow. The calf vascular resistance was also found to be higher during upright position compared to supine position. The authors concluded that transient orthostatic hypotension occurs in healthy individuals. Increasing heart rate, stroke volume and vascular tone was the counter regulatory mechanism to correct it. The Sino-atrial node is the intrinsic pace maker of the heart and it connects to the Atrio-ventricular node and bundle of His. The conducting system then divides into Purkinje system that innervates cardiomyocytes. Sympathetic nervous system increases the discharge of the conduction pathway to induce inotropic and chronotropic action. Such reflex counter-regulatory mechanism is controlled by thoraco-lumber region of the spinal cord. The mechanism is by activating sympathetic nervous outflow. Another study described sympathetic response contributed to increase in vasomotor tone at the time of orthostatic hypotension (Kelbaek, Gjorup et al. 1989). Ten young and healthy volunteers had tilt table testing in thermal neutral environment (20-24° C) after a light meal to study the sympathetic function. Clonidine, a central adrenergic blocker, was also given to assess the effect of absence of sympathetic function. When the arterial pressure dropped during head-up tilt, the cardiac output, heart rate and femoral blood flow rose briefly. Total vascular conductance followed the pattern exhibited by cardiac output. Clonidine blunted the reflex vasomotor responses to correct orthostatic hypotension. The effects of the counter regulatory mechanism began to be evident at 10 to 15 seconds and completed within 30 to 60 seconds after standing
In our study, post-prandial increase of heart rate after standing, systolic blood pressure at 120 minutes and mean arterial pressure at 60 and 120 minutes after meal were lower in PWS group suggesting blunted reflex sympathetic vasomotor response. Our finding supports the Di Mario et al and Purcell et al (DiMario, Bauer et al. 1993, DiMario, Dunham et al. 1994, DiMario and Burleson 2002, Purcell, Jenkins et al. 2013). Di Mario et al. studied ANS function in PWS examining respiratory sinus arrhythmia (DiMario, Bauer et al. 1993) and reported that the pulse rate was generally higher than the controls but was significantly less variable to the change in intra-thoracic pressure associated with breathing. The finding in our study is similar to Di Mario’s study. The pulse at recumbent position is higher than the controls and it is less variable to the orthostatic stress.

Interestingly, post-prandial plasma gastrin levels in PWS group were higher than controls. Both the post-prandial gastrin AUC $_{0-120 \text{ min}}$ and the median plasma gastrin level at 120 minutes post meal were significantly higher in PWS group than the controls. High gastrin level is commonly observed in human and animals after vagotomy. Vagotomy was the surgical treatment for peptic ulcer disease many years back before the discovery of the aetiological role of *Helicobacter pylori*. There are many papers that studied effect of vagotomy procedure on plasma gastrin in human and animal models. In a study, thirteen vagotomised rats and twelve sham operated rats were fed using intra-gastric infusion of liquid meal (Ami, Doi et al. 1993). Plasma gastrin levels increased after the liquid meal in both vagotomised and sham operated rats but the levels were significantly higher in the vagotomised rats. A similar study on vagotomised dogs also showed higher plasma gastrin level after meal (Eysselein, Niebel et al. 1985). The study also investigated the effect of Superior mesenteric and coeliac ganglionectomy disrupting sympathetic nervous supply to the stomach and small intestine on food related gastrin release. No effect of local sympathetic nerve disruption was noted on the gastrin release. Furthermore, Takeuchi et al studied male Sprague- Dawley rats and vagotomised animals had higher plasma gastrin level than sham surgery group (Takeuchi, Speir et al. 1980). Gastrin binding capacity as indicated by its receptor expression also increased in vagotomised animals. In another animal study, plasma gastrin level rose 12 weeks after the Proximal Gastric Vagotomy (Reina, Lopez-Cantarero et al. 1997). In another study, human patients who had had Vagotomy and Pyloroplasty for Peptic ulcer disease had higher plasma gastrin level than pre-operative levels (McGuigan and Trudeau 1972). The plasma gastrin level was reduced after Vagotomy and Antrectomy in the study as the numbers of the antral G- cells, which produce gastrin, were reduced after Antrectomy. The
authors explained that the reduction in gastric acid-pepsin secretion, and lack of Vagal inhibition might be the likely mechanisms by which gastrin was stimulated after Vagotony. Similarly, Busman et al compared the pre-Vagotomy and post-Vagotomy plasma gastrin levels in 262 patients (Busman, Brombacher et al. 1987). The mean gastrin level after highly selective Vagotomy was significantly higher than the pre-operative level.

PWS gene region contains Gamma Amino Buteric Acid (GABA) receptor genes (GABRB3, GABRA5, and GABRG3) (Cassidy and Driscoll 2009, Rout, Abdul-Rahman et al. 2012). Those genes are not imprinted and have bi-parental expression in normal individuals. Therefore genetic deletion of the region would reduce the expression of GABA-A receptor’s α5, β3 and γ3 subunits. GABA receptors are expressed in central nervous system, testes, heart, lungs, pancreas, intestine, kidneys and adrenal glands. Mice with GABA receptor β3 gene deficiency suffer from hypotonia and neuro-behavioural problems similar to PWS (Hashemi, Sahbaie et al. 2007). Using $[^{11}\text{C}]$ flumazenil (FMZ) positron emission tomography of the binding sites of benzodiazepine, Lucignani et al assessed the presence and number of cerebral GABA-A receptors in patients with PWS (Lucignani, Panzacchi et al. 2004). The patients had reduced $^{11}\text{C}$ FMZ binding predominantly in the cingulate, frontal and temporal cortices, and insula compared to unaffected participants of the study. The study concluded that there was altered GABA-A receptor composition or number in the cortical regions. A study supported the finding of Lucignani by reporting higher plasma GABA level in PWS (Ebert, Schmidt et al. 1997). The mean plasma GABA level in PWS and Angelman syndrome was two to three times higher than that of moderately obese and developmentally appropriate individuals or non-obese and developmentally delayed individuals. This finding confirmed that the GABA-A receptor configuration and its number were affected in PWS. Plasma GABA level is significantly higher and it exerts its effect on normal population of GABA-B receptors in PWS. In an animal study, stimulation of GABA-B receptors by Baclofen resulted in a clinical features similar to PWS such as reduced cognition, antinociception, muscle relaxation, decreased hormone production, increased food intake, and reduced intestinal peristalsis (Saffouri, Weir et al. 1980).

GABA is the cardinal neurotransmitter at the Nucleus Tractus Solitarius and C1 area of the brain stem. C1 neurons connect to thoracic spinal cord and control the sympathetic discharge to cardiovascular tissues and adrenal medulla (Yamada, Norman et al. 1982). Therefore, our findings of poor sympathetic nervous function after a mixed meal may be due to abnormal GABA-ergic action at NTS.
Since GABAergic system also has the inhibitory function, there may be poor inhibitory action of vagus nerve similar to post-vagotomy state. The plasma catecholamine levels were similar in both groups but the urinary catecholamine excretions were significantly higher in the PWS group. Plasma catecholamine levels are generally tightly regulated. Generally urinary catecholamine represents catecholamine production. In the PWS group, the urine levels were higher in both at the pre- and post- prandial periods. Since the first urine sample was collected after waking, it reflects the catecholamine production over night. Therefore, higher urinary catecholamine levels in PWS group is unlikely to be due to acute stress reaction. The main source of blood catecholamine is from the adrenal medulla but the spill over of those from the nerve cells is significantly less than adrenal origin. Adrenal medulla synthesizes noradrenaline and adrenaline from tyrosine. It is metabolized by catechol-O- methyl transferase (COMPT) or mono-amine oxidase (MAO), sulfated and excreted in urine, or re-uptake from the nerve endings. Adrenal catecholamine release is controlled by co-ordination of two input pathways. Pre-ganglionic sympathetic neuron innervates the adrenal medulla (de Diego, Gandia et al. 2008) and the intercellular gap junction stimulation among chromaffin cells (Guerineau and Desarmenien 2010). Mature sympathetic neurons synthesize brain- derived neurotrophic factor (BDNF) and pre-ganglionic neurons express full- length trkB BNDF receptors. BNDF supports the growth and survival of preganglionic sympathetic neuron that innervate the adrenal medulla (Schober, Wolf et al. 1998). Endogenous release of acetyl choline acting on the muscarinic receptors at the splanchnic-chromaffin cell synapse releases catecholamine from chromaffin cells (de Diego, Gandia et al. 2008). The gap junction communication accelerates when there is reduction in cholinergic synaptic transmission (Martin, Mathieu et al. 2001, Khasar, Green et al. 2003, Martin, Mathieu et al. 2003, Colomer, Martin et al. 2012). Among newborn rat, in which cholinergic synaptic transmission has not yet matured, there is increased gap junction stimulation between chromaffin cells (Martin, Mathieu et al. 2003). Moreover, chronic blockade of synaptic transmission after surgical denervation of the adrenal gland also resulted in increased dye coupling between chromaffin cells indicating increased communication. AO Martin et al. (Martin, Mathieu et al. 2003) blocked the post- synaptic nicotinic cholinergic receptor using Hexamethonium, Oxystillbene derivative F3 and snake neuro- toxin (α- bungarotoxin). Oxystillbene derivative F3 blocks the α3 subunit and α- bungarotoxin irreversibly antagonizes α7 subunit of the nicotinic acetylcholine receptors. When adrenal medulla slices were incubated with the nicotinic cholinergic blockers, the spontaneous trans- synaptic electrical potential decreased but the intercellular communication between chromaffin cells...
increased. Gap junction coupling probability, an indicator of inter-chromaffin cell communication, increased when hexamethonium and α-bungarotoxin, acetyl choline antagonists, were given to test animals (Colomer, Martin et al. 2012). Khasar et al described that vagotomised rats had higher plasma epinephrine (Khasar, Green et al. 2003, Colomer, Martin et al. 2012). When compared to sham operation, the animals that had sub-diaphragmatic vagotomy had higher plasma epinephrine since day 3 of surgery. Our finding of higher production of catecholamine despite attenuated central sympathetic nervous function, as shown in our cardiovascular results, indicates the condition is similar to post-vagotomy state. Together with our finding of higher post-prandial plasma gastrin level, higher urinary catecholamine levels support that in PWS, there is vagal dysfunction. Watkins et al studied effect of vagotomy in adult male Sprague-Dawley rats on the pain sensation. The hyperalgesic effect of cytokine interleukin 1β was blocked by the surgical sub-diaphragmatic vagotomy (Scott, Greenwood et al. 2013). The classical PWS phenotype of high pain threshold may be due to Vagus-mediated effect on nociceptors and it supports our finding of vagal dysfunction.

The median fasting plasma acyl ghrelin level in PWS group was higher than the control group. It got suppressed after the mixed meal and reached to similar level as controls after 120 minutes. The rate of decrease of plasma acyl ghrelin was faster in PWS than controls. This is a novel finding that contrasts previous publications which reported partial suppression of ghrelin after a meal in comparison with the healthy controls. Haqq et al performed an uncontrolled study on 11 PWS participants (Haqq, Stadler et al. 2003). In eleven PWS subjects, total plasma ghrelin after an overnight fast got suppressed after a mixed meal by 35%. Gimmenez-Palom and group did a small case-control study on 7 adult PWS patients and lean control group (Gimenez-Palom, Gimenez-Perez et al. 2007). The total plasma ghrelin was higher than the lean control group at fasting state and any time point after a liquid meal. The ghrelin AUC $0-6\ h$ remained higher in PWS compared to obese and lean controls. Similarly Paik et al reported higher total ghrelin before and after oral glucose load (Paik, Choe et al. 2006). In the study, eleven children with PWS and ten normal obese controls underwent oral glucose test after an overnight fasting. Fasting plasma level of acyl ghrelin was higher in PWS compared to the controls. The nadir of acyl ghrelin post glucose load was 30 minutes for PWS and 90 minutes for the controls. The post-glucose load remained higher in the PWS group at all-time points till 120 minutes post glucose load.
Our PWS participants were already on growth hormone therapy at the time of study and had plasma IGF1 level in normal to even marginally higher range. A number of studies studied growth hormone’s effect on acyl ghrelin level in PWS. It was shown to have no effect on plasma acyl ghrelin in a study (Hauffa, Haase et al. 2007). Using oral glucose tolerance test, Hauffa et al investigated fasting and post glucose load plasma total and acylated ghrelin before and after commencement of growth hormone therapy. The median duration of growth hormone therapy was 1.18 years. Plasma acyl ghrelin levels did not change during oral glucose tolerance test before and after the commencement of growth hormone therapy. However, total ghrelin level declined after growth hormone therapy in PWS cohort. This result was conflicted by a case- control study that compared the fasting total plasma ghrelin before and after growth hormone therapy (Feigerlova, Diene et al. 2008). The fasting total ghrelin level was not affected by the growth hormone therapy in children with PWS in the study. Hauffa et al later reproduced similar result to his previous study (Hauffa and Petersenn 2009). Total ghrelin level but not acyl ghrelin level decreased after commencement of growth hormone therapy in children with PWS. In fact, plasma acyl ghrelin was similar before and after the growth hormone therapy.

The fasting acyl ghrelin level in our PWS cohort is negatively correlated to the sympathetic nervous system function as indicated by percent change in pulse rate during fasting state. Autonomic nervous system has been thought to have a role on control of ghrelin. The exact mechanism of how ANS controls ghrelin remains unclear. It is very difficult to differentiate the sympathetic from para- sympathetic nervous functions, because ANS function is tightly controlled by reflex homeostasis and its effector organs are dually innervated.

2.8: Conclusion

Our data showed that there was defective autonomic nervous function in children with PWS, which in turn might have resulted in higher fasting plasma acyl ghrelin level. Our cardiovascular data suggested homeostatic sympathetic function was at fault in PWS. Our biochemical data suggested that there was poor vagal inhibition on gastrin and catecholamine productions complementing the cardiovascular findings. Although the fasting plasma acyl ghrelin level was high, there was more rapid acyl ghrelin suppression after the mixed meal in PWS. At 60 and 120 minutes post meal, the acyl ghrelin level was similar to the controls. Fasting percent change in PR at 30s was negatively correlated to the fasting acyl ghrelin level. Therefore, poor ANS function in PWS may cause higher fasting acyl ghrelin.
Chapter 3: Energy Expenditures in PWS

3.1: Introduction

3.1.1: Global obesity

Recent publication of the Australia Government indicated that one out of four children and three out of five adults are overweight or obese. There is 5% increase in prevalence of overweight or obesity since 1995 in Australia (Welfare 2013). Alike Australian data, obesity has been an escalating global burden (Ng, Fleming et al. 2014). It has been estimated to cause 3.4 million deaths, 3.9% loss of year of life, and 3.8% of disability-adjusted life-year worldwide (Ng, Fleming et al. 2014). The prevalence of overweight and obesity increased from 28.8% in 1980 to 36.9% in 2013 in adult men; and 29.8% in 1980 to 38.0% in 2013 in adult women worldwide in the study. Increased prevalence, not dissimilar to adults, is seen in children and adolescents especially in the developed countries. Currently the prevalence is 23.8% and 22.6% for boys and girls respectively in 2013 compared to 16.9% and 16.2% respectively in 1980 (Ng, Fleming et al. 2014). The prevalence of overweight and obesity in children and adolescents is also rising in developing countries. It was 8.1% in 1980 and increased to 12.9% in 2013 for boys, and similarly, 8.4% in 1980 for girls and 13.4% in 2013 (Ng, Fleming et al. 2014). The fundamental cause of general childhood obesity is energy imbalance as a result of increased intake of nutrient rich food items (Parsons, Power et al. 1999, Swinburn, Egger et al. 1999, Caballero 2002, Story, Sallis et al. 2009, Cameron, Ball et al. 2012, WHO 2014) and low level of physical activity (Schlicker, Borra et al. 1994, Goran and Sun 1998, Ravussin and Gautier 1999, Boreham and Riddoch 2001, Lazzer, Boirie et al. 2003). Alarmingly, the overweight and obese children worldwide grow into obese adults (Delpeuch and Maire 1997, Kotani, Nishida et al. 1997, Rossner 1998, Imamura, Izawa et al. 2004, Juonala, Raitakari et al. 2006, Moayeri, Bidad et al. 2006, Cheng 2007). The prevalence of obesity in children has continued to rise despite good public awareness of complications related to it.

3.1.2: Energy Expenditure

Energy production, heat production or thermogenesis is essential for life. Animal and human cells produce heat by oxidizing foodstuff such as glucose, fatty acid and amino acids.

Energy output can be generally divided in Basal Metabolic Rate, Activity related thermogenesis and Thermic effect of food. Because thermic effect of food intake is negligible, overall energy expenditure is dependent upon Basal Metabolic rate and Activity thermogenesis (Levine 2004, Levine 2004).
3.1.3: Resting Energy Expenditure or Basal Metabolic Rate

Basal metabolic rate or resting energy expenditure is the rate of energy production at rest in the post-absorbed state in thermo-neutral environment (Himms-Hagen 1984). It is essential for keeping cells alive. The energy consumed in contraction of heart and respiratory muscles during resting ventilatory effort constitute resting energy expenditure. Change in resting energy expenditure, either increase or decrease, is slow (Himms-Hagen 1984).

Mitochondria are the organelles of all living cells that generate heat or energy including resting energy expenditure (Prusiner and Poe 1968). The details of the mechanistic of energy production are explained below. Prusiner and Poe also pointed out that 75% of the energy produced from mitochondria is free energy and 25% is conserved as ATP (Prusiner and Poe 1968).

3.1.4: Activity Associated Energy Expenditure or Thermogenesis

Muscle activity is the major source of energy production in human. The skeletal muscle activities associated with exercise, non-exercise movement or shivering contribute to the activity-associated thermogenesis. As myocytes contract energy is dissipated. When a motor nerve is stimulated the muscle fibers depolarize resulting in release of Calcium from intracellular stores. It leads to activation of myosin ATPase and, using the energy released, myosin molecules slide on the actin causing shortening of myofibrils (Ganong 2001). Increased ADP supply from ATPase also accelerates mitochondrial oxidation, which further increases rate of combustion of fuel (Himms-Hagen 1976). The capacity of muscle to produce energy is not fixed. The muscles of trained athletes have better mitochondrial function and energy usage (Proctor, Sinning et al. 1995, Fernstrom, Tonkonogi et al. 2004). Morphometry of Vastus lateralis muscle of older individuals had decrease in size of the type II fibers responsible for fast twitch and oxidative capacity (Proctor, Sinning et al. 1995). In another study, biopsy specimens of lateral aspect of quadriceps muscle showed significant increase in mitochondrial respiration and adenine nucleotide translocase after six weeks of endurance training (Fernstrom, Tonkonogi et al. 2004). Mitochondrial content and oxidative capacity increase with exercise training. A sedentary muscle had only 65% of the electron transport chain complex content and lower maximum ATP production rate of an active muscle (Conley, Amara et al. 2013). Activity related thermogenesis can further divided into exercise associated (EAT) and non-exercise associated thermogenesis (NEAT) that is defined as energy expenditure related to activity other than volitional exercise (Levine 2004, Levine 2007). It includes fidgeting,
chewing gum, pacing around a room, toe tapping, shopping, dancing, gardening and so on. In industrialized countries the non-exercise associated thermogenesis is the most important component of total daily energy expenditure. Even in avid exercisers, non-exercise associated thermogenesis is the most predominant component of total daily energy expenditure. Low non-exercise associated thermogenesis due to mechanization of day-to-day life can lead to obesity.

3.1.5: Thermic Effect of Food
There is increase in energy expenditure over several hours after eating and it is called Thermic effect of food. It is due to utilization of ATP in the metabolic disposal of ingested food such as digestion, storage or waste disposal. For disposal of a molecule of glucose, 2 to 4 molecules of ATP is used resulting in 5 to 10% loss of caloric value of the ingested carbohydrate (Himms-Hagen 1976). For lipid, 2 molecule of ATP or 1.4 to 4.6% of the energy acquired by ingesting lipid is used for disposal of free fatty acid or triglyceride (Himms-Hagen 1976). As for protein, 4 molecules of ATP or 20 to 30% of the energy acquired from the ingested protein is used for disposal of amino acids (Himms-Hagen 1976). A small amount of energy is also used for transport of nutrients across intestinal wall (Schultz and Curran 1970).

3.1.6: Mitochondria
Mitochondria serve as generators of ATP and energy in every mammalian cell. Mitochondria are located in every cell of human and are involved in many cellular functions including production of energy and reactive oxygen species (Ganong 2001). Mitochondria have outer and inner membranes. The outer membrane is permeable to small metabolites but the inner membrane is controlled to maintain high electrochemical gradient created by the respiratory chain. It is important for energy production by oxidative phosphorylation. Nutrients are oxidized and yield electrons in the form of reduced Nicotinamide Adenine Dinucleotide (NADH) and Flavin Adenine Dinucleotide (FADH2). These reduced cofactors generate and transfer electrons to the electron transfer chain. The electron transfer chain activates pumping of protons across the inner membrane of mitochondria. The electron transfer chain comprises of five protein complexes on the inner membrane: complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (ubiquinone cytochrome c oxidoreductase), complex IV (cytochrome oxidase), and complex V (F1F0-ATP synthase). Complexes I, III and IV pump protons across the inner membrane as the electrons down the respiratory chain creating electrochemical potential gradient across
known as the proton motive force. The energy conserved from the membrane potential is used by the complex V to synthesize Adenosine triphosphate (ATP) from Adenosine diphosphate (ADP). The energy derived is then dissipated out and released as heat (Echtay 2007). In health, most adult produce approximately 65 kg of ATP per day powered by the mitochondrial respiratory chain (Rich 2003).

3.1.7: Mitochondrial Uncoupling Proteins
Mitochondrial membrane carriers facilitate specific exchange of molecules between cytosol of a cell and mitochondrial matrix to carry out metabolic pathways such as the citric acid cycle, fatty acid β oxidation, urea cycle, oxidative phosphorylation, and generation of heat by dissipation of the proton gradient. Uncoupling proteins (UCP) are members of mitochondrial carriers, and are proton carriers located on the inner membrane of mitochondria. UCPs have a tripartite structure that consist of three repeat domains each with two hydrophobic transmembrane α-helix regions spanning the mitochondrial inner membrane. UCPs have both the N- and C-terminal ends facing towards the cytosolic side of the inner membrane. UCP-1 is specific for brown adipose tissue (BAT) but also seen in longitudinal smooth muscle layers of digestive, uterine and male reproductive tracts (Nibbelink, Moulin et al. 2001). Recent literature proved that not only human neonates but children, adolescents and even adults bear brown adipose tissue around neck, interscapulæ area and axilla (Dawkins and Scopes 1965, Heaton 1972, Bouillaud, Combes-George et al. 1983, Lean and James 1983, Lean, James et al. 1986, Bouillaud, Villarroya et al. 1988). Therefore thermogenesis by the UCP1 at BAT is significant in human neonates, children, adolescents and adults.

UCP-2 is widely expressed in tissues including stomach, thymus, spleen, macrophages, hypothalamus and β cells of the pancreas (Azzu and Brand 2010). UCP-3 is primarily seen in skeletal muscles but also in brown adipose tissue and heart (Azzu and Brand 2010).

The uncoupling of such protein is stimulated by fatty acids releasing heat. UCP1 mediates proton leak and it dissipates the proton motive force. It in turn reduces the number of proton flowing through the ATP-synthase. The leak of proton through UCP-1 uncouples substrate oxidation from phosphorylation of ADP to ATP. It leads to faster oxygen consumption and energy derived from the oxidation of the substrate is released as heat (Cannon and Nedergaard 2004).

There are two classes of mitochondrial proton leak: basal and inducible (Thrush, Dent et al. 2013). Basal leak takes place in all tissues and no specific mechanism appears to
control the process (Thrush, Dent et al. 2013). It has been postulated that the basal leak facilitates energy expenditure by increasing sensitivity and decreasing the response time to change in the cellular ATP utilization (Rolfe, Newman et al. 1999). Inducible proton leak is catalyzed by the UCPs (Divakaruni and Brand 2011) and adenosine nucleotide translocase enzyme may be the control mechanism (Andreyev, Bondareva et al. 1988). Adenosine nucleotide translocase mediates ADP influx into mitochondrial matrix and efflux of ATP.

3.1.8: Role of ANS in controlling UCPs

Respiratory burst of UCP1 at brown adipose tissue increases in response to noradrenaline (Sluse, Jarmuszkiewicz et al. 2006). Brown adipose tissue is highly vascularized and richly innervated by the sympathetic nervous system. When BAT- bearing organisms such as human or grizzly bears get exposed to cold stress, sympathetic nervous system is activated and secretes noradrenaline (Smith and Horwitz 1969). When noradrenaline binds to $\beta_3$ adrenoreceptors, it stimulates cyclic AMP- dependent liberation of intracellular free fatty acids, which in turn is a signal for activation of UCP1- induced proton leak and energy production (Jezek 2002). Moreover, activation of $\alpha_1$ adrenoceptor by noradrenaline potentiates the thermogenic function of $\beta_3$ adrenoceptors (Zhao, Cannon et al. 1997).

3.1.9: Role of sympathoadrenal system in Energy Expenditure

Since 1950s it has been clearly shown that ANS has an important role in energy expenditure (Hsieh and Carlson 1957, Hsieh, Carlson et al. 1957). The authors demonstrated that cold- acclimatized rats with intact ANS but treated with curare, to inhibit shivering, maintained body temperature when the environmental temperature was lowered from 30°C to 5°C. It meant the thermogenesis or energy expenditure was not affected in those animal with intact ANS even though shivering was prevented. However, when the animals were given hexamethonium, an ANS ganglion blocker, thermogenesis was completely inhibited and the body temperature fell. Administration of noradrenaline restored the thermogenesis and prevented the drop in body temperature (Hsieh and Carlson 1957, Hsieh, Carlson et al. 1957). The uncoupling protein deficient mice consumed less oxygen after treatment with beta- 3 adrenergic receptor agonist and they are sensitive to cold exposure (Enerback, Jacobsson et al. 1997).

Both sympathetic nervous system and adrenal medulla are stimulated during cold stress to increase thermogenesis (Therminarias, Chirpaz et al. 1979, Landsberg and Young 1983, Landsberg and Young 1984). The afferent neurons arising from the peripheral (skin) and
central (hypothalamus, and brain stem) relay thermal senses to anterior and posterior hypothalamus. The efferent fibers increase the sympathetic tone and in turn causing vasoconstriction, shivering, piloerection, and energy production (Gale 1973, Landsberg and Young 1983, Landsberg and Young 1984).

In an animal study, it was shown that food induced thermogenesis involves sympathetic nervous system activation on BAT. The study also found that adrenergic drive increased resting oxygen consumption and inter- scapular BAT mass but a beta- adrenergic blocker, Propranolol, decreased the resting oxygen consumption (Landsberg and Young 1984). Noradrenaline is synthesized and stored in sympathetic nerve endings and upon stimulation; it is released to act on the post- synaptic adrenergic receptors. Adrenaline is the circulating hormone of the adrenal medulla. It is released into the blood stream for physiological functions. Hypothalamus and brain stem regulates the sympathetic adrenergic outflow.

Mature sympathetic neurons synthesize brain- derived neurotrophic factor (BDNF) and pre-ganglionic neurons express full- length trkB BNDF receptors. BNDF supports the growth and survival of preganglionic sympathetic neuron that innervate the adrenal medulla (Schober, Wolf et al. 1998).

3.1.10: Role of hypothalamo- pituitary- thyroid axis in control of energy expenditure

Hypothalamo- pituitary- thyroid axis is also important in regulating energy homeostasis. Thyroid hormone influences energy production through its action on the hypothalamus effecting appetite, thermogenesis, locomotion and autonomic regulation (Lechan and Fekete 2006). Thyrotropin from hypothalamus stimulates anterior pituitary to secrete thyroid stimulating hormone which in turn regulates thyroid hormone synthesis from the thyroid gland. Thyroid hormone is important in sustaining body’s core temperature (Silva 1995, Silva 2001, Bianco, Salvatore et al. 2002, Silva 2003). It increases thermogenesis by stimulating numerous metabolic pathways involved in development, remodeling, and delivery of energy to all tissues (Silva 1995). Thyroid hormone also interacts with sympathetic nervous system in generating facultative thermogenesis. When thyroid hormone function is completely blocked, resting energy expenditure can be reduced as much as 30% and homeostatic thermogenesis in response to cold exposure can be dampened (Silva 2003). On the other hand, hyperthyroidism increases energy expenditure by increasing ATP consumption (Silva 2003). Thyroid hormone has control on uncoupling proteins of mitochondrial inner membrane (Gong, He et al. 1997, de Lange, Lanni et al. 2001, Lebon, Dufour et al. 2001). De Lange et al. administered T3 to hypothyroid rats and
demonstrated there was increased expression of uncoupling protein 3 in skeletal muscles resulting in increased resting metabolic rate (de Lange, Lanni et al. 2001). Gong at al. quantified the uncoupling protein 3 in skeletal muscles (Gong, He et al. 1997). In hypothyroid rats, UCP3 decreased three folds whereas hyperthyroid rats had 6 folds higher. In a human study, healthy adults were given T3 for three days and it resulted in 70% rise in the rates of ATP synthesis and tri-carboxylic acid cycle flux in skeletal muscles which was detected by nuclear magnetic resonance (Lebon, Dufour et al. 2001). Thyroid hormone also acts on uncoupling protein 1 in brown adipose tissue (Enerback, Jacobsson et al. 1997, Ribeiro, Carvalho et al. 2001, Bianco, Salvatore et al. 2002). Hypothyroid mice treated with T3 or thyroid hormone receptor beta selective ligand were found to have restored uncoupling protein 1 in brown adipose tissue between scapulae (Ribeiro, Carvalho et al. 2001). Subsequently, the hypothyroid mice, after being treated with T3 and infused with norepinephrine, had 3 degree Celsius elevation in body temperature but not in the THR beta ligand. Ribeiro et al. concluded that thyroid hormone stimulates UCP1 in BAT and augments adrenergic responsiveness in thermogenic tissues (Ribeiro, Carvalho et al. 2001).

3.1.11: EE in PWS

In growth hormone naïve patients with PWS, resting metabolic rate and average metabolic rate were found to be low in a study (Butler, Theodoro et al. 2007). However, the duration of the study was only 8 hours on each participants and the result may not represent the actual EE. When adjusted for lean body mass the TEE and REE are not different. Other studies found that the basal metabolic rates and activity associated energy expenditure were higher than the controls after being adjusted for the lean body mass in patients with PWS who were not on growth hormone therapy (van Mil, Westerterp et al. 2000, Goldstone, Brynes et al. 2002). Having obesity and relatively higher RMR (van Mil, Westerterp et al. 2000, Goldstone, Brynes et al. 2002) suggests that the activity associated energy expenditure must be definitely low in patients with PWS. EAT may be insignificant because patients with PWS rarely exercise (Davies and Joughin 1993, van den Berg-Emons, Festen et al. 2008). Therefore NEAT represents activity-associated thermogenesis in PWS and it is very likely to be low. ANS dysfunction and probably hyperghrelinæmia may be the causes of low NEAT in PWS.


3.1.12: Gap in knowledge
Specific metabolic rate such as total, or resting or basal energy expenditures have been studied in PWS but not on the complete profile of all components of EE. Moreover, the role of ANS and ghrelin in EE in PWS remains unknown.

3.2: Clinical Question and Hypothesis
Since obesity is a common problem in PWS, the EE in children with PWS is expected to be low. A study showed lower unadjusted resting energy expenditure but another study showed better resting energy expenditure if adjusted for fat free mass. Having obesity and relatively higher REE for lean body mass in PWS patients suggest that the problem lies in the activity associated thermogenesis. Since most children with PWS have reduced physical activity (Davies and Joughin 1993, van den Berg-Emons, Festen et al. 2008), NEAT appears to be the main contributor of the activity associated thermogenesis. Since NEAT is dependent upon ANS and ghrelin status, it is biologically possible that NEAT is low in patients with PWS. This led to my hypothesis that the NEAT is lower in PWS than the controls which cannot be compensated by the relatively high REE for lean body mass.

3.3: Aim, study type and populations
The aim of the study is to compare the total and differential EE, measured during three consecutive days among PWS patients and controls. PWS group comprised of genetically confirmed patients. 48 PWS patients who attended the PWS clinic at Mater Children’s Hospital were approached personally and by post. The first 16 agreed to participate were included in the study. There were 2 PWS participants of mixed Asian and Caucasian descent. The rest of PWS and the controls are Caucasians. One PWS patient with type 2 diabetes mellitus and one who was on psychotropic drug were excluded. Volunteers without features of and diagnosis of PWS were included as controls. Friends and siblings of PWS patients, and children who attend general endocrinology and obesity clinic were approached. Due to difficulty in recruiting controls, normal weight controls were not included in the study.

Growth hormone therapy in PWS cohort will be a confounder and should be an exclusion criterion. However, with the commencement of Australian pharmaceutical benefit scheme’s growth hormone therapy in all PWS patients, it would be difficult to recruit PWS participants who are not on growth hormone. Hence it may pose as a weakness of the study. Further exclusion criteria include any hypothalamic disorders, stimulant and psychotropic drugs use.
3.4: Statistics (EE study)

The primary outcomes of the study are total and differential energy expenditures. A sample size of 16 in each group will allow a difference of 1 SD of energy expenditure between the two groups to be detected, if it exists (see statistics section of chapter 2). The energy expenditures are adjusted to both the body weight (Wt) and fat free mass (FFM) or lean mass for comparison between PWS and control groups. Log-log regression method was used for this purpose (Davies, Cole et al. 1989, Davies and Cole 2003). The energy expenditure was expressed as Kcal/ kg Wtp or FFMp. Logarithm value of energy expenditure; weight and FFM were calculated first. The log value of energy expenditure and log value of either weight or FFM were regressed. The value of p is the slope of the linear regression between either log of weight or FFM, and log of energy expenditure. By adjusting energy expenditure to Wtp or FFMp, it can be compared across different weight or body composition.

For the purpose of measuring NEAT, the definition by Levine (Levine 2004) was used. A period of exercise was arbitrarily defined as “Sustained high energy expenditure with high physical activity level and heart rate for 30 minutes or more”. Available data suggests that children with PWS had reduced physical activity compared to unaffected children (Davies and Joughin 1993) in addition to muscle hypotonia (Prader, Labhart et al. 1956, Eiholzer 2005, Cassidy and Driscoll 2009) and skeletal abnormalities (West and Ballock 2004, Kroonen, Herman et al. 2006). Thence, children with PWS generally are not able to participate in usual sporting events or exercise programs that commonly last for 60 minutes or more. To give children with PWS who participated in the study a sporting chance, only thirty minutes or more of high and sustained physical activity was defined as an episode of exercise.

For comparison of energy expenditures adjusted to weight and FFM in PWS and control groups, Student t-test is used. SPSS statistics version 22 for Mac (IBM Analytic Software) was used to perform statistical tests. Student t tests was used to compare the means of the cardiovascular data in which two tail p value of <0.05 to reject the null hypothesis.
Actiheart is 7 mm thick with a diameter of 33 mm and it houses a movement sensor a rechargeable battery, a memory chip and electronics (Brage, Brage et al. 2005). A wire, as shown in Figure 13, runs to a smaller clip that is placed around apex of the heart by means of an electrocardiograph adhesive pad. Actiheart measures acceleration, heart rate, heart rate variability and ECG amplitude. Actiheart measures heart rate and body movement to calculate energy expenditure (Crouter, Churilla et al. 2008). The memory chip can store data of EE and physical activity index of an epoch of 1 minute for more than 11 days. During physical activity, movement sends a voltage signal that is converted to a binary signal quantifying acceleration as numerical levels. It measures ECG signals that is electronically amplified by a factor of 900. It is converted to heart rate (beats per minute) and written to the memory at the end of each epoch. The measurable heart rate is between 31 to 250 beats per minute according to the manufacturer. 

Actiheart has been validated as a tool to measure energy expenditure (Brage, Brage et al. 2005, Brage, Brage et al. 2006, Butte, Wong et al. 2010, De Bock, Menze et al. 2010, Adolph, Puyau et al. 2012, Campbell, Prapavessis et al. 2012, Villars, Bergouignan et al. 2012). Crouter and group validated Actiheart against Cosmed K4b (2) and the former provided similar calculation to the latter in different physical activities (Crouter, Churilla et al. 2008). In a study, heart rate measurement by R-wave impulses using Actiheart agreed with electrocardiograph during rest and treadmill locomotion (Brage, Brage et al. 2005). There was strong linear relationship between movement and acceleration in the study. However, further studies are needed to validity in other activities and free-living. Adolph et al. validated actiheart, actical, RT3 accelerometer against room calorimetry. The authors found that the devices had similar accuracy for sedentary, light, moderate to vigorous
levels of physical activity (Adolph, Puyau et al. 2012). Furthermore, Butte et al. validated actiheart against doubly labeled water in children (Butte, Wong et al. 2010). The Bland-Altman plot in the study showed the value of total energy expenditure is in good agreement with doubly labeled water with no systematic bias. In addition, another study found that lean and sedentary adults, tested for heart rate variation and physical activity using actiheart, had a good level of agreement in energy measurement with doubly labeled water (Villars, Bergouignan et al. 2012).

All participants in our study were requested to have usual diet and physical activity for 24 hours before the study. They presented to Mater Children’s Hospital in the morning for the testing. Then they had body composition measured using bio-impedance (Bodystat). Afterwards Actiheart was placed on the anterior chest wall with an aid of an electrocardiograph pad. Participants wore Actiheart for three days. Children with PWS can have disturbing skin picking if an adhesive ECG pad is placed on the chest for a long time. Therefore, to obtain average EE over a long period, it was decided to measure EE using Actiheart for three days. Actiheart measures physical activity index, total EE and differential EE. After three days of recording energy expenditures, the device was uploaded to obtain the total and differential metabolic rates of the participants. The Actiheart estimated REE and AAT. Energy spent during exercise period was deducted from AAT to measure NEAT. The exercise periods were identified according to the definition described above and the energy used during the period was subtracted from the total activity associated thermogenesis to calculate for non-exercise associated thermogenesis as defined by Levine. The total and differential metabolic rates will then be adjusted for weight and lean body mass and be compared between PWS participants and controls.

3.6: Ethical Approval

The study has been approved by the HREC of the University of Queensland (Reference No. 2010000367) and Mater Health Services (Reference No. 1654C).

3.7: Results

16 children with PWS and 16 controls were recruited to the study. The median age of the PWS cohort was 9.32 years (Inter quartile range 5.29) and due to difficulty in recruiting matched controls, older volunteers were accepted. As a result, the controls’ median age was 12.16 years (inter quartile range 6.12, p = 0.078). The PWS group comprised of 16 genetically confirmed individuals (nine females and seven males) and the control group
had 16 volunteers (six females and 10 males). Apart from three of the controls, no other
participants in both PWS and controls were in puberty. Three of the PWS participants were
on stable thyroxine therapy for central hypothyroidism. Upon download, there was no
Actiheart recording for three of the PWS participants. This is probably due to poor contact
of the electrode to the skin likely due to the habitual skin picking in PWS patients. The
PWS participants are shorter compared to the controls. The median height Z score for
PWS group was -0.39 (IQR 1.45) while that of the controls was 1.03 (IQR 1.61, p 0.049).
The weight Z score in PWS group was 1.05 (IQR 1.62) and for the controls, 1.26 (IQR
1.32). The difference between groups did not reach statistical significance (p value of
0.54). The median BMI Z score in PWS group was 1.50 (IQR 1.39) and the control group
was 1.10 (IQR- 1.11, p = 0.423). The lean mass in significantly lower in PWS group
compared to the control group (26.00%, IQR 12.48 and 44.84%, IQR 20.85, p= 0.013).
Similarly, waist and height ratio as an indicator of adiposity, trends to be higher in PWS
cohort. The median waist and height ratio in PWS group was 0.55 (IQR 0.27) and that of
the control group was 0.5 (IQR 0.1, p = 0.055). The PWS participants were treated with
growth hormone and had plasma IGF1 level within normal range in female PWS
participants and slightly higher than normal upper limit in males. The mean (SD) plasma
IGF1 level for the whole group was 48.7 (28.2) nmol/l. The mean (SD) IGF1 level for male
PWS participants was 47.6 (29.5) nmol/l (reference range: 34.9-45.2 nmol/l) and that of
female participants was 47.6 (28.7) nmol/l (reference range: 33.2- 54.4).

### Table 15: Comparison of characteristics of the PWS and control groups

<table>
<thead>
<tr>
<th></th>
<th>PWS</th>
<th>Control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Yr) Median (IQR)</td>
<td>9.32 (5.29)</td>
<td>12.16 (6.12)</td>
<td>0.078</td>
</tr>
<tr>
<td>Height Z score Median (IQR)</td>
<td>-0.39 (1.45)</td>
<td>1.03 (1.61)</td>
<td>0.049</td>
</tr>
<tr>
<td>Weight Z score Median (IQR)</td>
<td>1.05 (1.62)</td>
<td>1.26 (1.32)</td>
<td>0.545</td>
</tr>
<tr>
<td>BMI Z score Median (IQR)</td>
<td>1.50 (1.39)</td>
<td>1.10 (1.11)</td>
<td>0.423</td>
</tr>
<tr>
<td>Waist to height ratio Median (IQR)</td>
<td>0.55 (0.27)</td>
<td>0.50 (0.1)</td>
<td>0.055</td>
</tr>
<tr>
<td>Lean mass % Mean (SD)</td>
<td>26.00 (12.48)</td>
<td>44.84 (20.85)</td>
<td>0.013</td>
</tr>
</tbody>
</table>
Total energy expenditure is strongly correlated to body weight and fat free mass ($r= 0.91$ and 0.86 respectively, $p< 0.001$ in both models). Similarly resting energy expenditure is positively correlated to weight and fat free mass ($r= 0.935$ and 0.94 respectively, $p< 0.001$ in both models). Correlation between activity associated energy expenditure and weight or fat free mass did not reach statistical significance. The correlation coefficient was 0.31 for activity associated energy expenditure and weight ($p= 0.13$) and 0.18 for fat free mass ($p= 0.38$). Non-exercise associated thermogenesis and weight is positively correlated and the coefficient is 0.39 ($p= 0.048$) and for fat free mass is 0.26 ($p= 0.19$).

Moreover, total energy expenditure and resting energy expenditure are positively correlated to age of the participants ($r= 0.72$ and 0.80, $p< 0.001$ for both). Both activity associated and non-exercise associated thermogenesis are not significantly correlated to age ($r= 0.13$ and 0.16, $p= 0.54$ and 0.43 respectively).
Figure 18: Comparison of EE adjusted for weight by log-log regression in PWS and control groups. The Y axis is weight adjusted EE (Kcal/kg) and X axis is body weight (kg). Figure A: TEE, Figure B: REE, Figure C: AAT and Figure D: NEAT.
Figure B:
Figure C:
Figure D:
Figure 19: Comparison of EE adjusted to fat free mass by log-log regression in PWS and control groups. The Y axis is FFM adjusted EE (Kcal/kg) and X axis is fat free mass. Figure A: TEE, Figure B: REE, Figure C: AAT and Figure D: NEAT.
Figure B:

![Graph showing REE/FFM^p vs FFM (Kg)]
Figure C:
Figure D:

![Graph showing comparison of weight adjusted EE in PWS and control groups.](image)

Table 17:
Comparison of weight adjusted EE in PWS and control groups. Mean value in Kcal/kg (Standard Deviation). *p<0.01 and **p<0.02 difference between 2 groups

<table>
<thead>
<tr>
<th></th>
<th>PWS</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEE/ Wt&lt;sup&gt;p&lt;/sup&gt;</td>
<td>287.93* (32.12)</td>
<td>432.58 (38.66)</td>
</tr>
<tr>
<td>REE/ Wt&lt;sup&gt;p&lt;/sup&gt;</td>
<td>183.05** (12.62)</td>
<td>200.48 (18.07)</td>
</tr>
<tr>
<td>AAT/ Wt&lt;sup&gt;p&lt;/sup&gt;</td>
<td>98.20* (41.21)</td>
<td>296.87 (97.22)</td>
</tr>
<tr>
<td>NEAT/ Wt&lt;sup&gt;p&lt;/sup&gt;</td>
<td>87.96* (42.08)</td>
<td>221.58 (79.94)</td>
</tr>
</tbody>
</table>
Table 18:
Comparison of fat free mass adjusted EE in PWS and control groups. Mean value in Kcal/kg (Standard Deviation) *p<0.01 for difference between 2 groups

<table>
<thead>
<tr>
<th></th>
<th>PWS</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEE/FFM^p</td>
<td>364.47* (51.05)</td>
<td>560.80 (62.72)</td>
</tr>
<tr>
<td>REE/FFM^p</td>
<td>216.25* (16.53)</td>
<td>249.92 (21.76)</td>
</tr>
<tr>
<td>AAT/FFM^p</td>
<td>135.27* (59.26)</td>
<td>416.07 (140.26)</td>
</tr>
<tr>
<td>NEAT/FFM^p</td>
<td>122.16* (62.95)</td>
<td>235.25 (88.99)</td>
</tr>
</tbody>
</table>

The total daily energy expenditure, REE, AAT and NEAT adjusted to weight are lower in the PWS group than the control group. The mean (SD) TEE adjusted for weight was 287.93 (32.12) Kcal/kg in PWS group and 432.58 (38.66) Kcal/Kg for the control group (p<0.01). The mean (SD) REE adjusted for weight for PWS group was 183.05 (12.62) Kcal/Kg whereas that of control group was 200.48 (18.07) Kcal/kg (p<0.001). Both the weight adjusted overall activity associated energy expenditure and non-exercise associated thermogenesis were lower in PWS group compared to the controls [96.20 (41.21) Kcal/kg vs. 296.87 (97.22) Kcal/kg, and 87.96 (42.08) Kcal/kg vs. 221.58 (79.94) Kcal/kg respectively] (p<0.01).

When the energy expenditures were adjusted for the fat-free mass, lower total and differential energy expenditure were again observed in PWS group. The mean (SD) TEE adjusted for fat-free mass in PWS group was 364.47 (51.05) Kcal/Kg and in control group was 560.80 (62.72) Kcal/kg (p<0.01). The mean (SD) fat-free mass adjusted REE was 216.26 (16.53) Kcal/Kg in PWS and 249.92 (21.76) Kcal/Kg in control group (p<0.01). When adjusted for fat-free mass, the activity associated energy expenditure was 135.27 (59.26) Kcal/Kg in PWS group and 416.07 (140.26) Kcal/Kg in control group (p<0.01). The non-exercise associated thermogenesis in PWS group was 122.16 (62.95) Kcal/Kg and 235.25 (88.99) Kcal/kg in control group (p<0.01). Using our arbitrary definition of exercise, no participant in PWS group had any recording of physical activity that met the definition.
Correlation between fasting acyl ghrelin and energy expenditures does not show any relation. The correlation coefficients between fasting acyl ghrelin and TEE is -0.054 (p=0.87), REE is -0.1 (p=0.76), AAT is 0.031 (p=0.92), and NEAT is 0.056 (p=0.86). The correlation coefficients between percent change in pulse rate at 30 sec after standing, a marker for sympathetic nervous function, and EE are as follow. With TEE is 0.13 (p=0.69), REE, -0.05 (p=0.87), AAT, 0.32 (p=0.32), and NEAT, 0.30 (p=0.35).

3.8: Discussions
Similar to the available evidence (Bernstein, Thornton et al. 1983, Garby, Garrow et al. 1988, Welle and Nair 1990, Cunningham 1991, Goran and Sun 1998), our data showed that the total and resting energy expenditure were positively correlated to weight and fat free mass. Although positive correlation of non-exercise associated thermogenesis and weight was statistically significant, the correlation between fat free mass was not statistically significant. The overall TEE, REE, AAT and NEAT in PWS group when adjusted to both body weight and fat free mass were lower than the control group. Better REE or activity-associated EE have been reported in PWS (van Mil, Westerterp et al. 2000, Goldstone, Brynes et al. 2002) but no data is available for in NEAT, or TEE adjusted for weight or fat free mass in PWS. To our knowledge, our findings are the first to compare adjusted NEAT, AAT and TEE of PWS patients to unaffected individuals.

It has been proven that growth hormone therapy improves the energy expenditure and body composition (Carrel, Myers et al., Sode-Carlsen, Farholt et al., Brambilla, Bosio et al. 1997, Nakazato, Murakami et al. 2001, Carrel, Myers et al. 2002, Mogul, Lee et al. 2008). Despite of the growth hormone therapy in PWS participants achieving optimal level of plasma IGF1, the energy expenditures remained low in our PWS group. An animal study showed that when rats were treated with intracerebroventricular injections of ghrelin, NPY and Argp there was significant reduction in locomotor activity of the animals compared to the controls that received vehicle (St-Pierre, Karelis et al. 2004, Tang-Christensen, Vrang et al. 2004). However, there was no significant correlation between fasting acyl ghrelin and activity associated or non-exercise associated thermogenesis. The relation between TEE, REE, AAT and NEAT, and percent change of pulse rate on standing for 30 sec at fasting state did not reach statistical significance. Nevertheless, the autonomic nervous system has crucial role in controlling thermogenesis and our findings of dysautonomia in PWS may play a role in low EE. A larger sample size
than 16 participants in each cohort is needed to identify significant correlation between ANS function and EE.

Dysautonomia in PWS patients may have impaired the mitochondrial functions as ANS has been shown to have influence mitochondrial functions (Smith and Horwitz 1969, Zhao, Cannon et al. 1997, Jezek 2002, Sluse, Jarmuszkiewicz et al. 2006). In an animal study using electron microscope, PWS model mice with imprinting centre defect were found to have abnormal mitochondrial proliferation, swollen and disorganized mitochondria with disrupted membrane in skeletal and cardiac muscles, compared to the littermates (Yazdi, Su et al. 2013). Moreover, the gene expression in the study showed the PWS mice had up-regulated Mrpl15 (mitochondrial ribosomal protein L15) and down-regulated Alas1 (aminolevulinic acid synthase 1) genes in brain tissues. Mrpl15 is responsible for mitochondrial protein synthesis (Kenmochi, Suzuki et al. 2001) and Alas1, rate limiting and first enzyme in haem biosynthesis (Mason, Fan et al. 2009). In the muscle tissues of the PWS mice, Yazdi et al. discovered down-regulation of Bdh (3-hydroxybutyrate dehydrogenase), and Mte1 (mitochondrial acyl-co A thioesterase 1) (Yazdi, Su et al. 2013). Both genes are involved in mitochondrial fatty acid oxidation and are important for energy production. Acadl (acetyl-coenzyme A dehydrongenase, long-chain) was also down regulated in PWS mice muscle compared to their littermates in the study. Acadl belongs to the acyl-CoA dehydrogenase family of mitochondrial flavoenzymes involved in fatty acid and branched chain amino acid metabolism. In the PWS-IC model, there are abnormal mitochondrial genes, abnormal mitochondria and mitochondrial proliferation. Therefore, at least in PWS-IC mice model, there is evidence that energy production is low as a result of mitochondrial abnormalities (Yazdi, Su et al. 2013). A case report described a two-year-old girl with PWS who has a novel mutation of MTCYB gene that is responsible for mitochondrial complex III deficiency (Yis, Ezgu et al. 2014). MTCYB is mitochondrial cytochrome b gene, which is not part of PWS gene region. The affected PWS patient in the report had hypotonia, hepatosteatosis, and episodes of lactate acidosis for which she was treated with Carnitine and Coenzyme Q 10 (CoQ-10). CoQ-10 is a lipid soluble component of cell membranes which is responsible for electron transfer from complex I and II to complex III on the inner mitochondrial membrane (Bentinger, Tekle et al. 2010). It is important for mitochondrial function and energy production. There is no concrete evidence suggesting CoQ-10 treatment is beneficial in PWS (Eiholzer, Meinhardt et al. 2008). Butler et al. measured plasma CoQ-10 and reported it was lower in PWS compared to the lean controls but not different to obese controls (Butler, Dasouki et al. 2003). Another study also reported blood CoQ-10 level was not different to obese and lean
controls. But PWS group had higher carnitine esters and ester- to- free carnitine ratios than the controls (Miller, Lynn et al. 2011). Carnitine is a cofactor for the transport of fatty acids into the mitochondria for β- oxidation. High ester- to- free carnitine ratio occurs when mitochondrial energy production is impaired (Calvani, Reda et al. 2000).

Another probable reason for low energy expenditure is the long non- coding RNA Snord116 in PWS gene region (Zhang, Bouma et al. 2012, Powell, Coulson et al. 2013, Powell, Coulson et al. 2013). Its expression was prominent in hypothalamic regions that control bodily homeostasis such as food intake, blood pressure and regulate energy balance (Zhang, Bouma et al. 2012). Snord116 expression was found to be highly in para- ventricular nuclei, ventro- medial nuclei and particularly very high in the Arcuate nuclei which are important feeding circuitry. Zhang et al. speculated that Snord116 might be the underlying cause for the phenotype of PWS. It was supported by a case report of an individual with a micro- deletion of snoRNA region of 15q11.2 who has features of PWS (Sahoo, del Gaudio et al. 2008). If it is proven to be true, PWS will be the first human genetic condition to prove that non- coding RNAs are as important as protein coding RNAs as Mattick hypothesized some years ago (Mattick 2005). In another study, Powell et al. used fluorescence in- situ hybridization probes targeting the splice junction of IncRNA such as 116HG on mice brain. It revealed distinct nuclear clouds and it was observed on hypothalamus as well as forebrain, hindbrain, cortex, hippocampus, and cerebellum (Powell, Coulson et al. 2013). Snord116 deleted mice, when compared to the littermates, were found to have abnormal gene important for signal transduction and metabolic regulation such as MTOR (Mechanistic target of Rapamycin). Brain slices were stained for mTOR protein and there was up-regulation of mTOR in cortex but not in hippocampus. Circadian clock regulation in hypothalamic nuclei, such as supraoccular nuclei, controls mTOR activity (Cao, Anderson et al. 2011). Powell’s study did not prove the pathogenicity of snord116 in PWS but provided the novel explanation of loss of snord116. It will be very interesting to identify the RNAs generated from the snord116 locus absent in PWS.

Our data showed low energy expenditures captured on Actiheart among PWS participants who inherently have hypotonia (Prader, Labhart et al. 1956, Eiholzer 2005, Cassidy and Driscoll 2009), sedentary life style (Davies and Joughin 1993) and a degree of skeletal abnormality (West and Ballock 2004, Kroonen, Herman et al. 2006). Being inactive can cause poorer mitochondrial functions. Exercise is known to increase mitochondrial oxidative capacity and contents (Zoll, Sanchez et al. 2002). Zoll et al. studied human skeletal muscle’s mitochondrial respiration functions among athletic, active, and sedentary individuals. The athletic individuals had better mitochondrial respiration rate, oxidative
function and more efficient coupling. The authors compared and found that ADP-stimulated mitochondrial respiration before exercise ($\dot{V}_0$) was similar in all groups but after exercise ($\dot{V}_{\text{max}}$) it was significantly higher in athletic group indicating better mitochondrial respiration. Moreover, $\dot{V}_{\text{max}}$ was positively correlated to $\dot{V}_{O_2\text{max}}$. The study also examined kinetics of ADP ($K_m$ constant value) in skeletal mitochondria. Athletic individuals had higher value of $K_m$ indicating better mitochondrial oxidative function. In animal studies (Veksler, Kuznetsov et al. 1995, Kuznetsov, Tiivel et al. 1996), higher $K_m$ was seen in oxidative muscles and low value in glycolytic muscles. Since individuals with PWS do not exercise regularly, their mitochondria would not have good respiration rate, oxidative function or efficient coupling resulting in poor energy expenditure.

In summary, PWS group had lower total, resting and activity associated energy expenditure in our study. Dysautonomia may be the cause for impairment in mitochondrial function resulting in lower resting and non-exercise associated energy expenditures leading to lower total energy expenditure. Non-coding RNA ($Snord116$) from the PWS region and fasting hyperacylgrehlimaemia may have a role in impairment of mitochondrial functions. Sedentary nature of children with PWS may also have contributed to the poor mitochondrial functions and low energy expenditure as a result.

3.9: Conclusions
We found that the TEE, REE, AAT and NEAT were lower in children with PWS compared to the controls. Despite of the growth hormone therapy, which generally increases energy expenditure in PWS patients, the overall energy output was found to be lower than the controls. To our knowledge our study is the first to examine TEE and NEAT in PWS. A number of possible causes such as dysautonomia, high fasting plasma acyl ghrelin level, $snord116$ gene and mitochondrial abnormalities suggested by PWS-IC deleted animal model may have influence on low energy expenditure in participants with PWS. Our PWS cohort had low EE and low lean mass although being treated with GH. They will benefit from higher GH dose and increased physical activity such as regular exercise and incidental activity (NEAT).
Chapter 4: Conclusions

We conclude that there is dysautonomia in PWS. Our cardiovascular data suggests poor sympathetic nervous function; and the gastrin and catecholamine data pointed to poor vagal function. There are two probable explanations for poor sympathetic outflow and poor vagal functions. Firstly, reduced GABA-A receptor number and probably its action in PWS as a result of the deleted β3, α5, and γ3 subunit genes of GABA-A receptor; and secondly, probable exaggerated GABA-B action due to compensatory hyper-γ- amino- butyric-acidaemia and normal GABA-B receptors. GABA is the key neurotransmitter between Nucleus Tractus Solitarius and C1 neurons that connect to the thoracic spinal cord that sends efferent neurons to sympathetic ganglions. GABA is generally an inhibitory neurotransmitter and GABA dysfunction may be the cause of poor vagal inhibitory function that lead to high post-prandial plasma gastrin production, and increased catecholamine production from adrenal medulla probably by increased chromaffin cells gap junction communications.

Poor sympathetic and parasympathetic nervous functions per se can lead to obesity. Bray et al. proved Mona Lisa hypothesis which means “ Most obesities known are low in sympathetic activity” (Bray 1991). Bray et al. published a number of studies on relationship between sympathetic nervous system and food intake (Bray 1991, Bray and York 1998, Bray 2000) and reported that there is inverse relation between sympathetic nervous system and food intake. Findings of Bray et al. were supported by further reports of acute and chronic increase in sympathetic outflow, as a result of later hypothalamic lesion, had led to weight loss in the study animals (Arase, Sakaguchi et al. 1987). Monda et al. reported pre-menopausal women with poor ANS function, based on heart rate variability, were obese (Monda, Messina et al. 2006). Therefore our finding of dysautonomia itself can cause increased food intake or energy intake, and obesity in PWS.

Dysautonomia may also have a causal role in our finding of high fasting plasma acyl ghrelin in PWS participants. To our knowledge, rapid reduction in plasma acyl ghrelin among PWS participants after meal to similar levels to the control is a novel finding. Since acyl ghrelin in PWS participants is well suppressed after a meal, their poor satiation may be not be induced by acyl ghrelin.

In our study, the PWS participants had lower total, resting, activity associated, and non-exercise associated energy expenditure compared to the controls. Despite the adequate growth hormone therapy that maintained appropriate blood IGF1 level, the PWS participants had lower energy expenditure. The growth hormone dosage for PWS patients
recommended by Australian Pharmaceutical Benefit Scheme is lower than the dosage used elsewhere in the world. It may be the reason for our finding of low energy expenditure in PWS unlike previous reports of growth hormone treated PWS patients having resting energy expenditure similar to controls. To our knowledge, we are the first to report lower total energy expenditure and non-exercise associated thermogenesis in the PWS participants than the controls. Again dysautonomia in PWS participants may have caused low resting and non-exercise associated energy expenditure. High fasting acyl ghrelin and snord116, a long non-coding RNA present in the PWS gene region, may also have contributed to it. Animal model of PWS, PWS-IC deleted mice, had abnormal mitochondrial proliferation; swollen and disorganized mitochondria with disrupted membrane, and abnormal expressions of mitochondrial genes. However, no such findings have been replicated in human model or other PWS mice models.

Our finding of dysautonomia and high fasting acyl ghrelin may be one of the causes of increased food intake or increased energy intake in PWS participants. Despite of growth hormone therapy, the energy expenditures of PWS participants were lower than the controls. Hence, there is imbalance between energy gained and energy utilized which leads to obesity in PWS. GABA dysfunction as a result of attenuated GABA-A receptor action and probable increased GABA-B receptor function, at the level of Nucleus Tractus Solitarius may be the underlying pathological cause of imbalance between energy gain and expenditure which leads to development of obesity in PWS.

**Strengths of the study**

Most of the available literatures on ANS in PWS were based on cardiovascular functions. Our research studied not only the cardiovascular functions but also the production of gut hormone gastrin and adrenal catecholamine to assess ANS functions in PWS. We also report ANS data after stimulating by a mixed meal. Therefore our data provides more comprehensive ANS profiles in PWS.

We used a protease inhibitor in plasma sample preparation for measurement of acyl ghrelin. It reduced the chance of degradation of acyl ghrelin by protease and allowed more accurate measurement of the peptide. To our knowledge, the post-prandial suppression of acyl ghrelin to a level similar to the controls at 60 and 120 minutes post meal is a novel finding.

We report adjusted total, resting, activity associated and non-exercise associated energy expenditures in children with PWS. Our data describes comprehensive nature of thermogenesis in children with PWS.
Limitations

Our study has a number of limitations. Government subsidized growth hormone therapy became available for all children under 18 years with PWS before the recruitment. Therefore, all PWS participants were already on growth hormone therapy at recruitment which may be a limitation of our study. Due to difficulty in recruiting controls, older participants were accepted as controls. Actiheart data from 3 PWS participants were lost likely due to poor contact of the electrode as a result of skin picking on the adhesive tapes on the chest wall. The number of participants may not be enough to show any correlation between ANS functions, acyl ghrelin levels and energy expenditures in our study. Our sample size of 16 may be under-powered to show the correlation between ANS function and EE.
Chapter 5  
Future Directions  
5.1: Plasma GABA level  
PWS gene region contains expression of subunits that make up gamma amino butyric acid receptor A (Wagstaff, Knoll et al. 1991) and positron emission tomography scan showed GABA- A receptor number in brain is low in patients with PWS (Lucignani, Panzacchi et al. 2004). Plasma GABA level was significantly higher in patients with PWS supporting deficiency of GABA-A receptors (Ebert, Schmidt et al. 1997). The historical evidence of dysfunction of GABA system in PWS can explain our finding of dysautonomia and higher fasting acyl ghrelin in children with PWS. We plan to measure GABA level in the leftover fasting blood sample to complement our current findings.

5.2: Ghrelin auto-antibody in PWS  
In our findings, despite the fact that children with PWS had higher fasting acyl ghrelin which got suppressed to a level similar to the controls at 60 and 120 minutes after meal, their waist-to-height ratio was higher and lean body mass was lower. High fasting acyl ghrelin may stimulate appetite but its post-prandial levels did not support the typical poor satiation and hyperphagia seen in PWS (Prader, Labhart et al. 1956, Holland, Treasure et al. 1993). I further hypothesize that there must be a contributory mechanism or different mechanism controlling appetite in PWS other than ghrelin level alone.

Recently autoantibody against ghrelin was discovered in healthy human and in those with anorexia nervosa (Fetissov, Hamze Sinno et al. 2008, Terashi, Asakawa et al. 2011). Fetissov et al. identified IgG and IgA against leptin, ghrelin, peptide YY, and neuro-peptide Y in healthy women. In another study, anti-ghrelin IgG was identified and it was mainly an immune complex with desacyl ghrelin in patients with anorexia nervosa (Terashi, Asakawa et al. 2011). Auto-antibodies for acyl ghrelin were decreased and it resulted in higher unbound acyl ghrelin suggesting phenomenon of ghrelin resistance in malnourished patients with anorexia nervosa. The role of anti-ghrelin autoantibody on food intake was tested in an animal study (Takagi, Legrand et al. 2013). Free feeding rats received intra-peritoneal injection of ghrelin alone or together with anti-ghrelin antibodies from obese, anorexic and lean participants. Rats that received ghrelin and anti-ghrelin antibodies from obese subjects had increased food intake. Furthermore, the authors tested whether the ghrelin IgG prevents ghrelin degradation. When ghrelin alone is incubated at 37°C, its concentration decreased significantly indicating rapid degradation but when incubated
together with ghrelin IgG extracted from either controls, anorexics, or obese patients, most of the level initially added could be detected in-vitro.

GABA- A receptor subunits RNA expression was noted in murine peripheral macrophages and modulates their IL 6 and IL 12 production (Reyes-Garcia, Hernandez-Hernandez et al. 2007). Moreover, GABA-A receptors mediate inhibitory response on T lymphocytes (Tian, Chau et al. 1999). Having deficiency of GABA- A receptors in PWS, there may be increased risk of poor immune surveillance and autoimmunity (Tian, Lu et al. 2004).

Therefore, it is my hypothesis that in PWS, due to GABA- A receptor deficiency, there is poor T cell immune surveillance. It increases risk of formation of ghrelin auto- antibody which prevents degradation of orexigenic ghrelin that in turn stimulates appetite. I have a plan to measure and compare ghrelin IgG in leftover PWS and control samples.

5.3: Nesfatin-1

Nucleobindin 2 (NUCB2)- encoded satiety and fat- influencing protein 1 (nesfatin-1) inhibits food intake upon injection to ventricles in animal studies (Oh, Shimizu et al. 2006, Shimizu, Oh et al. 2009). Only the Mid- segment of nesfatin-1 contains potent anorexigenic effect compared to N- segment or C- segment (Shimizu, Oh et al. 2009). The anorexigenic effect of mid- segment of nesfatin-1 is independent of leptin pathway as reduced food intake was still observed in db/db mice and overfed obese mice (Shimizu, Oh et al. 2009). Central injection of nesfatin-1 increases c-fos expression in Paraventricular nucleus of hypothalamus and Nucleus Tractus Solitarius.

In another animal study using immunohistochemistry, co-localization of ghrelin and nesfatin-1 was observed in hypothalamus and anterior intestine of the goldfish (Kerbel and Unniappan). In the same study, intracerebroventricular injection of nesfatin-1 and ghrelin reduced the mRNA expression of pre-proghrelin and NUCB2 in hypothalamus respectively, indicating reverse relationship between the two peptides (Kerbel and Unniappan). Maejima et al. elegantly described nesfatin-1 regulates oxytocinergic signaling in hypothalamus and Nucleus Tractus Solitaries which in turn stimulates melaoacortin pathway to induce anorexia independent of leptin (Maejima, Sedbazar et al. 2009).

Swaab et al. performed post- mortem examination on PWS brain and reported that the number of oxytocin containing neurons is low (Swaab, Purba et al. 1995, Swaab 1997). Therefore, it is my hypothesis that in PWS, anorexigenic Nesfatin-1 may be low resulting in reduced oxytocin expression in hypothalamus and poor satiation. To date, little is known
about plasma profile of nesfatin-1 in relation to a meal in healthy human or nesfatin-1 status in PWS where satiety is not commonly achieved after a meal.
I plan to measure the fasting plasma Nesfatin-1 levels by enzyme linked immunoassay and compare the levels between the PWS and control groups. A financial grant has been obtained already and an ethics application is in progress for the projects.
References


Appendix 1
Benefit of Early Commencement of Growth Hormone Therapy in Children with Prader-Willi Syndrome

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ABSTRACT

Prader-Willi syndrome (PWS) is a chromosomal disorder and growth failure is a common presentation. Growth hormone (GH) treatment is beneficial in PWS although the optimal age for starting GH is unknown. We investigated whether GH response in PWS was associated with the age of GH commencement by comparing 16 children who commenced GH before 3 years of age (early group) with 40 children who commenced GH after 3 years of age (late group) from the Ozgrow database. Height SDS, body mass index (BMI) SDS, bone age (BA)-chronological age (CA) ratio, change in height (ΔHt) SDS and change in BMI during 4 years of GH treatment were compared between the groups. The early group had better height SDS and ΔHt SDS. BA delay was more pronounced in the early group but BA did not mature beyond CA with GH therapy in either group. Although the initial GH dose for the early group was lower than that of the late group, the former had better height outcome. The starting GH dose seen in the database is lower than the dose used by international centres.

KEY WORDS

Prader-Willi syndrome, PWS, growth hormone, GH, benefit, Ozgrow

INTRODUCTION

Prader-Willi syndrome (PWS) is a genetic disorder characterised by growth failure, hypogonadotropic hypogonadism, hypotonia, sleep-related breathing disorders, developmental delay, behavioural problems, hyperphagia and obesity. It is due to loss of imprinted gene expression from the paternal chromosome 15q11-q13 region. Normally paternally inherited genes in this region are expressed while maternal genes are inactivated. Loss of expression most frequently occurs due to paternal deletion of this region (70% of PWS) or less frequently due to maternal uniparental disomy of chromosome 15 (25% of PWS). The remaining 5% of PWS is caused by other structural abnormalities on chromosome 15, such as microdeletion within the PWS imprinting gene centre.

Patients with PWS have impaired growth hormone (GH) responsiveness during stimulation testing with GH releasing hormone and arginine, and numerous studies have described the benefits of GH therapy in this syndrome. Children with PWS treated with GH (1 mg/m²/day) for 2 years demonstrated normalisation of height standard deviation score (SDS), faster growth in head circumference, increased lean body mass accrual and decreased body fat together with improved language and cognitive functions. In another study GH was continued for a total of 4 years in three cohorts receiving different doses of GH. The benefit on growth velocity, body composition (lean body mass) and resting energy expenditure was noted with higher doses of GH (7 mg/m²/wk and 10.5 mg/m²/wk) but not with a dose of 2.1 mg/m²/wk. Bone mineral density, however, improved in all studied doses of GH. Moreover, GH improved sleep-related breathing disorders in children with PWS in a study in which 19 out of 25 patients showed improvement in the Apnoea/Hypopnoea

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Index (AHI) and Central Index (CI), but not of the Obstructive Index (OI) when polysomnography 6 months after commencement of GH was compared to that of baseline17.

However, in a mortality review in patients with PWS, the majority of sudden death was related to respiratory pathology18,19 and this finding was also supported by a study of KIGS, the Pfizer International Growth Database20.

Aim and hypothesis

Few published studies have investigated the optimal age for starting GH therapy in children with PWS. The aim of this study was to determine whether GH responsiveness was related to age at commencement of GH. Our hypothesis was that the earlier GH is started, the better the linear growth and body composition compared to starting GH therapy at older age. The biological rationale is that GH sufficient children under 3 years of age have greater height velocity. If children with PWS younger than 3 years of age receive GH early, this height velocity can be restored. Earlier commencement of GH may lead to better body composition because of earlier lean body mass accrual. It may in turn result in higher energy expenditure since lean tissues such as muscles have very active metabolism.

METHODS

We performed a retrospective analysis on growth data from the Ozgrow database of Australia and New Zealand regarding children with PWS on GH therapy. The Ozgrow database was established by the Australasian Paediatric Endocrine Group (APEG) in an attempt to collect data pertaining to GH therapy in children in Australia and New Zealand. GH therapy in Australia and New Zealand is subsidised by the federal governments and all applications for GH for various indications are captured and entered into the database.

The eligibility criteria for GH therapy according to the Department of Health and Aging of the Australian Government are short stature (height less than the first percentile as judged from the World Health Organisation International References for Growth which is based on data produced by the Centers of Disease Control, U.S. Department of Health and Human Services) and growth velocity less than the 25th percentile for bone age; or biochemical GH deficiency, that is peak GH level less than 10 mU/l in two challenge testings, plus a growth velocity less than the 25th percentile. There were no New Zealand patients included in this report.

The GH prescribers, who are paediatric endocrinologists or experienced general paediatricians, provide the diagnoses for short stature at the application for GH. The database records the diagnoses supplied by the GH prescribers as Ozgrow diagnostic codes. Growth data of children with the diagnostic code of ‘Dysmorphic and Genetic Syndromes – Prader-Willi Syndrome’ were extracted from the database.

The children with PWS who had GH treatment or are currently receiving GH were analysed. Those with no growth data for a minimum of 6 months or who did not qualify for GH therapy were excluded from the analysis. Sixteen patients (8 males and 8 females) who commenced GH before 3 years of age (early group [EG]) and 40 (24 males and 16 females) who commenced after 3 years of age (late group [LG]) were included in the study. Data up to 4 years after GH commencement were analysed.

The age- and sex-specific SDSs were calculated for height and body mass index (BMI) using the Centers for Disease Control 2000 reference data. Change in height SDS (Δ height SDS) after 1, 2, 3 and 4 years of GH therapy was calculated from baseline to assess the progressive nature of linear growth; and change in BMI SDS (Δ BMI SDS) was calculated for each year as a marker, albeit weak, for change in body composition21,22. The Ozgrow database records annual bone age assessment using the Greulich and Pyle method. The ratio of bone age to chronological age (BA:CA), which represents skeletal maturation in relation to chronological age, was calculated for each year.

Growth responsiveness was compared between EG and LG. The mean values of height SDS, BMI SDS, BA:CA, Δ height SDS, and Δ BMI SDS for each year up to 4 years of GH therapy were used in the comparison.
Statistical analysis

For statistical analysis, two sample t-test and Mann-Whitney U test were used for comparing means of the two groups; and linear regression for effect of age at commencement and height SDS at baseline on improvement of height SDS at the end of 4 years. All statistical procedures were performed using SPSS 15.

RESULTS

Baseline values of height SDS, BMI SDS, BA:CA and starting GH dose for the two groups are shown in Table 1. Prior to commencement of GH therapy, height SDS in the two groups was similar but BA:CA of the EG was less than that of LG, that is, the bone age delay was greater in the EG (p = 0.0006). The mean starting GH dose was

| Table 1 |
|-----------------|-----------------|-----------------|-----------------|
| Comparison of variables in the early group (EG) and late group (LG) before commencing GH therapy |
|               | Early group n = 16 | Late group n = 40 | p              |
| Male:Female   | 8.8              | 24.16            | 0.1260         |
| Ht SDS before GH therapy | -2.68 (0.76) | -2.77 (0.75) | 0.6700         |
| BMI SDS before GH therapy | -0.25 (1.8)  | +0.67 (1.77) | 0.0900         |
| BA:CA before GH therapy | 0.53 (0.19)  | 0.75 (0.18)  | 0.0006         |
| GH dose at commencement (mg/m²/wk) | 4.50 (0.97)  | 5.10 (1.14)  | 0.0700         |

Values of growth data and GH dose are shown as means (SD).

| Table 2 |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Results of height SDS, BMI SDS and BA:CA [mean (SD)] with GH therapy in the early group (EG) and late group (LG) |
| Measurement     | Group 1st year | Group 2nd year | Group 3rd year | Group 4th year |
|                 | EG   | LG   | p    | EG   | LG   | p    | EG   | LG   | p    | EG   | LG   | p    |
| Height SDS      |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 1st year        | -1.61 | -2.17 | 0.020 | -0.37 | 0.56 | 0.07 | 0.53 | 0.76 | 0.0000 |     |     |     |
|                 | (0.67) | (0.75) |     | (0.83) | (1.72) |     | (0.22) | (0.15) |     |     |     |     |
|                 | 16   | 40   |     | 13   | 39   |     | 12   | 38   |     |     |     |     |
| 2nd year        | -1.12 | -1.91 | 0.002 | -0.14 | 0.71 | 0.13 | 0.72 | 0.78 | 0.0330 |     |     |     |
|                 | (0.66) | (0.75) |     | (1.18) | (1.76) |     | (0.20) | (0.21) |     |     |     |     |
|                 | 15   | 33   |     | 12   | 32   |     | 12   | 30   |     |     |     |     |
| 3rd year        | -0.71 | -1.68 | 0.001 | 0.23 | 1.16 | 0.07 | 0.71 | 0.86 | 0.0023 |     |     |     |
|                 | (0.66) | (0.84) |     | (1.21) | (1.55) |     | (0.17) | (0.16) |     |     |     |     |
|                 | 13   | 29   |     | 12   | 28   |     | 11   | 23   |     |     |     |     |
| 4th year        | -0.70 | -1.54 | 0.003 | 0.73 (1.38) | 1.41 (1.83) | 0.31 | 0.82 | 0.89 | 0.0484 |     |     |     |
|                 | (0.59) | (0.72) |     | (0.16) | (0.17) |     |     |     |     |     |     |     |
|                 | 11   | 19   |     | 10   | 18   |     | 10   | 20   |     |     |     |     |

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Comparison of change in mean Ht SDS in EG and LG

Fig. 1: Comparison of mean Δ height SDS with each year of GH therapy in the early group (EG) and late group (LG). *p = 0.001, ** p = 0.002.

Comparison of mean Ht SDS in EG and LG

Fig. 2: Comparison of mean height SDS with each year of GH therapy in the early group (EG) and late group (LG). * p = 0.020, ‡ p = 0.002, # p = 0.001, † p = 0.003.

Comparison of mean BMI SD in EG and LG

Fig. 3: Comparison of mean BMI SDS with each year of GH therapy in the early group (EG) and late group (LG).

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Fig. 4: Comparison of mean BA:CA with each year of GH therapy in the early group (EG) and late group (LG). *p = 0.0006, *p = 0.0000, #p = 0.0330, †p = 0.0023, ‡p = 0.0484.

Fig. 5: Relationship between Δ height SDS at the first year of GH treatment and age of commencement.
lower in the EG compared to that of the LG. The mean Δ height SDS, mean height SDS, BMI SDS and BA:CA between EG and LG for each year of GH therapy are shown in Table 2 and Figures 1-4. The Δ height SDS and mean height SDS were greater in the EG compared to the LG (Figs. 1, 2, Table 2). Regression analysis showed age of commencement significantly influenced height SDS after 4 years of GH therapy (p = 0.003), whereas height SDS at baseline was not significantly related (p = 0.178). Linear regression of Δ Ht SDS at the first year of GH therapy and age of commencement of GH therapy for all the study population showed a negative relationship (R² = -0.316, p <0.0001) (Fig. 5). BA delay was more pronounced in the EG and it did not mature beyond CA in either group (see Table 2 and Fig. 4). A BMI SD for 4 years of GH therapy did not reach statistical significance (Fig. 3).

DISCUSSION

Our findings showed that improved linear growth was associated with commencement of GH before 3 years of age in children with PWS. Although height SDS before GH therapy was similar, after 4 years of GH therapy, the EG had achieved height SDS of -0.70 (0.59) while the LG achieved -1.54 (0.72) (p = 0.003). This clinical benefit is also associated with a benefit in health economics as smaller total doses of GH for smaller surface area are required for improved growth outcomes in children less than 3 years of age.

The mean BMI of the LG was greater than that of the EG (see Table 2 and Fig. 3) and it may be due to poor feeding in younger children with PWS. BMI increased with time even with GH therapy (Fig. 3) but in this study it was not possible to differentiate whether the increase in BMI was related to increase in fat mass or lean mass. Many studies have reported that body composition improved (decreased percent body fat and increased lean body mass) with GH therapy (7-6 mg/m²/week) in children with PWS compared to untreated children. As body composition is not routinely recorded in the Oszarov database, BMI SDS was used as an approximation. In our study, it was not possible to compare the BMI of our GH treated groups with PWS patients who did not receive GH therapy.

Bone age assessments in the database are made by different observers from various centres and it is difficult to standardize, but our BA:CA findings were consistently linear in both groups (see Fig. 4). BA was more advanced in the LG before the commencement of GH therapy and this phenomenon has been described in the PWS literature. Although BA increased in both groups, it did not mature beyond chronological age. Height potential, indicated by BA:CA, did not appear to be compromised by starting GH early.

From our data it was found that the starting GH dose used in the PWS literature was greater than the current Australian practice. It was not possible to extrapolate whether a higher starting dose in our cohort might result in better growth outcome or increased development of adverse effects.

No adverse effects related to GH therapy in PWS, such as death, deterioration of sleep-related breathing disorders, scoliosis, diabetes mellitus and central adrenal failure, are recorded in the Oszarov database. Since GH therapy for children with PWS is subsidised by the Australian Pharmaceutical Benefit Scheme, it is highly recommended that a central mechanism should be established to monitor such adverse effects in children with PWS undergoing GH therapy.

CONCLUSION

Within the limitations of a retrospective study, our findings support the early commencement of GH therapy in children with PWS. A prospective study is needed to confirm such a benefit. The height potential is not compromised by starting GH early in PWS. As is evident from the literature, the starting GH dose in current Australian practice for children with PWS is lower than in international centres. Furthermore, a central monitoring mechanism for adverse effects of GH in PWS is recommended in Australia.
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Normal Cortisol Response on Low-Dose Synacthen (1 μg) Test in Children with Prader Willi Syndrome


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Introduction: It has been postulated that central adrenal insufficiency (CAI), resulting from hypothalamic dysfunction, may contribute to the increased unexplained death rates in Prader Willi syndrome (PWS). A study using the overnight metyrapone test reported a 60% prevalence of CAI in children with PWS. We used a low-dose Synacthen test to screen for CAI in children with PWS.

Methods: We studied 41 children with genetic diagnosis of PWS [20 males; mean age, 7.68 (± 5.23) yr] in five pediatric endocrinology centers in Australasia. All participants were randomly selected, and none had a history of Addisonian crisis. Ten of the cohort were receiving sex hormone therapy, 19 were receiving GH, and four were receiving T3. Their mean body mass index z-score was 1.48 (± 1.68). Baseline morning ACTH and cortisol levels were measured, followed by iv administration of 1 μg Synacthen. Post-Synacthen cortisol levels were measured at 30 min, and a cortisol level above 500 nmol/liter was considered normal.

Results: The mean baseline ACTH and cortisol were 15 (± 14) ng/liter and 223 (± 116) nmol/liter, respectively. The mean 30-min plasma cortisol was 690 (± 110) nmol/liter, and the average increase from baseline was 201%.

Conclusions: Our result suggests that CAI is rare in children with PWS. (J Clin Endocrinol Metab 95: E646–E647, 2010)

Prader Willi syndrome (PWS) is a chromosomal disorder characterized by hypotonia, obesity, short stature, hypogonadotropic hypogonadism, behavioral problems, and sleep-related breathing disorders (1, 2). PWS is commonly due to paternal deletion or uniparental disomy of chromosome 15q11-13 (3–5), whereas a small percentage of PWS is due to imprinting center defect (6). Many of the typical features of PWS can be explained by hypothalamic dysfunction, and this hypothesis is supported by functional imaging studies (7–10).

There have been reports of unexplained deaths in patients with PWS (11–13). Most deaths were thought to be related to a combination of GH therapy, disordered breathing, and respiratory infections. Central adrenal insufficiency (CAI) due to hypothalamic dysfunction is a plausible alternative explanation that may have caused
contribute to the unexplained deaths. A study reported a 60% prevalence of CAI among children with PWS based on the result of an overnight metyrapone test (14). An earlier mortality report of eight children and two adults with PWS, who had unexplained death, demonstrated less than normal adrenal weight on postmortem examination, suggesting adrenal atrophy probably secondary to CAI (15).

Because of the recent evidence and its potential fatal complications, we screened for CAI in children with PWS in Australia. Rather than using an overnight metyrapone test, we have used a low-dose Synacthen test (LDSST) (1 μg Synacthen test) to screen for CAI. LDSST examines the stress response of the adrenal cortex to a low dose of synthetic ACTH. The adrenal cortex requires sufficient endogenous ACTH to maintain its ability to mount a stress response during Syndracthen tests. LDSST is considered to be robust, sensitive, and practical screening test for assessment of ACTH deficiency (16–18). In two studies, LDSST was found to be equally sensitive, if not more sensitive than the standard Synacthen test (SST; 250 μg Synacthen) and insulin-induced hypoglycemia (IHH) (16, 17). Abda et al. (16) studied 64 children with proven or suspected pituitary disease and compared the cortisol responses on LDSST and SST to IHH, and LDSST to SST. Using IHH as a standard and the cortisol cut-off level of 500 nmol/liter, the sensitivity of LDSST was 100%, and the specificity was 93.3%. The sensitivity and specificity of SST against IHH were 100% and 90%, respectively. The authors concluded that LDSST can replace SST or IHH for assessment of the hypothalamic-pituitary-adrenal (HPA) axis in patients with ACTH deficiency (16). Watson et al. (17) compared the cortisol responses on IHH, SST, and LDSST in children with pituitary disease with or without impaired HPA axis, as well as cortisol responses on SST and LDSST in the control group. In the study, both SST and LDSST were found to be equivalent to IHH in screening for integrity of the HPA axis. Furthermore, in a meta-analysis, LDSST was found to be superior to SST in diagnosing chronic CAI (18). In the study, cortisol area under the curve in LDSST was larger than that of SST.

**Subjects and Methods**

Our study was undertaken because of the publication that suggested high prevalence of CAI in PWS and its potential fatal complication (14). Forty-one children from five pediatric endocrinology units in Australia and New Zealand were included in this study. All children were genetically confirmed to have PWS and were randomly selected. The LDSST was performed in the morning on children who were inpatients for the day of testing. All children had rest for not less than 30 min while waiting for anesthetizing topical cream to be effective before venipuncture. An indwelling iv catheter was inserted, and the baseline blood sample for ACTH and cortisol was taken. Then 0.1 ml of Synacthen (250 μg in 1 ml) was drawn and added to 24.9 ml of normal saline. Special care and attention were paid to ensure that only 0.1 ml of Synacthen was drawn from the vial. One milliliter of the diluted solution (1 μg of Synacthen) was administered, followed by a 3-ml flush of normal saline. The blood samples for cortisol were collected at 30 min after Synacthen, and a serum cortisol level above 500 nmol/liter was considered a normal response (16). The serum cortisol cut-off level of 500 nmol/liter was shown to have 100% sensitivity and 93.3% specificity against IHH (16). The cut-off level, although arbitrary, is based on previous studies and is widely accepted.

ACTH and cortisol were measured by the respective laboratories at the different centers. ACTH was measured by either Immulite 1000 or Immulite 2000 (Siemens, Los Angeles, CA). Cortisol was measured by the Access (Beckman Coulter, Fullerton CA) and Architect (Abbott Diagnostics Division, Llantris, Longford, Ireland) in Queensland, Immulite 1000 and Immulite 2000.

(Siemens) in New South Wales; and E170 (Roche, Basel, Switzerland) in Auckland. Results from the RCQA Quality Assurance Program (RCQA Quality Assurance Program Pty. Limited, Chemical Pathology QAP Group, Endocrine Program Cycle 32, 6 July to 23 November 2009) do not show significant biases between these cortisol methods.

Results

The mean age was 7.68 (± 5.23) yr for the 41 patients, and 20 of them were male. The mean body mass index (BMI) z-score was +1.48 (± 1.68), and 10 of the patients were in early puberty induced by sex hormone replacement therapy. Nineteen of them were on GI, and four were on T4 at the time of testing. None had a past history of adrenal crisis, and none were treated with glucocorticoid replacement. The mean baseline serum ACTH level was 13 (± 14) ng/liter (normal range, 9–50) (Fig. 1) and 223 (±116) nmol/liter (normal range, 159–700), respectively. At 30 min after Synacthen, the mean serum cortisol level was 690 (± 114) nmol/liter, and the average increase of serum cortisol from the baseline was 201% (Fig. 2). All children had serum cortisol higher than 300 nmol/liter at 30 min after Synacthen.

Discussion

Our result, using LDSST, showed that the cortisol response in children with PWS is normal in the entire cohort, indicating that there must have been sufficient ACTH secretion to maintain health of the adrenal cortex. Our finding is a contrast to the recent finding of 60% prevalence of abnormal metyrapone test response in children with PWS (14). In the study, the authors examined the diurnal salivary cortisol levels in children with PWS. The level of salivary cortisol 30 min after wake-up in the CAI group was similar to the non-CAI group, which was consistent with a normal adrenocortical response to the physiological stress of waking up. The possibility is that the definition of an abnormal test in the overnight metyrapone test in the study was not based on changes in Blood D1-deoxycorticosteroid but on ACTH level, which is an unstable compound (19). In the study, seven patients had evidence of CAI because 11 deoxycorticosterone levels were less than 280 nmol/liter. However, unlike LDSST, the overnight metyrapone test assesses the whole HPA axis. It is therefore possible that some of our patients may have recently had ACTH deficiency but produced enough cortisol to have falsely normal results on LDSST.

The ACTH levels at baseline are variable in our cohort, depending on the time in the morning when the testing was done (Fig. 1). It is also possible that the abnormality lies in the other aspects of control of the HPA axis, such as circadian rhythm disturbance. It is supported by an animal study. Mice, like 2 gene, which falls in the PWS region, and null mice have defective circadian rhythm as well as symptoms similar to PWS (20).

Conclusion

Our study showed normal cortisol response to stress by using LDSST in all of the randomly selected children with PWS. Our result contradicts the 60% prevalence of CAI in children with PWS, and further testing is needed to evaluate the difference in prevalence—for example, an overnight metyrapone test or insulin-induced hypoglycemia on those who had normal response to LDSST to confirm the prevalence of CAI.

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Central sleep-disordered breathing and the effects of oxygen therapy in infants with Prader-Willi syndrome

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ABSTRACT

Objectives To describe breathing patterns in infants with Prader-Willi Syndrome (PWS), as well as the effects of supplemental oxygen (O2) on breathing patterns. Children with PWS commonly have sleep-disordered breathing, including hypopnoea and obstructive sleep apnoea, as well as central sleep breathing abnormalities that are present from infancy.

Design Retrospective cohort study

Patients Infants with a diagnosis of PWS.

Setting Tertiary children’s hospital.

Interventions Infants with PWS underwent full polysomnography, and in those with frequent desaturations associated with central events, supplemental O2 during sleep was started and followed with regular split-night studies (periods in both air and O2).

Results Thirty split-night studies on 10 infants (6 females aged 0.06–1.79 median: 0.68, IQ: 0.45, 0.78 years were undertaken. At baseline (i.e., air), children with PWS had a median (QR) central apnoea index (CAI) of 4.7 (1.9, 10.6) per hour, with accompanying falls in oxygen saturation (SpO2). O2 therapy led to statistically significant reductions in CAI to 2.5/hour (p=0.002), as well as a reduced central event index (CAI) and improved SpO2. No change in the number of obstructive events was noted. Central events were more prevalent in rapid eye movement sleep.

Conclusions It is concluded that infants with PWS may have central sleep-disordered breathing, which in some children may cause frequent desaturations. Improvements in CAI and CE as well as oxygenation were noted with O2 therapy. Longitudinal work with this patient group would help to establish the timing of onset of obstructive symptoms.

BACKGROUND

Prader-Willi syndrome (PWS) can be inherited either due to a deletion part of the paternal chromosome 15 or via uniparental disomy of the maternal chromosome 15—a phenomenon known as genomic imprinting. With the advent of genetic testing, there has been increased awareness of the condition, as well as earlier diagnosis. The estimated prevalence for the Australian population is 1:25,000.2

The presence of sleep-related breathing disorder in infants is well recognized.4–6 In addition to the obstructive sleep apnoea and hypopnoea reported with the condition, central respiratory control problems are noted in children with PWS.9,10 This is thought to be due to abnormalities in central chemoreceptor sensitivity in children. There is evidence from rodent and in vivo human studies to support this.8,14

There is very limited information available on the sleep-related breathing disorder in infants and very young children, and the primary aim of this study was to study the frequency of central apnoea during sleep in infants with PWS. Secondary aims were to describe the effects of supplemental oxygen on central apnoea indices and oxyhaemoglobin saturations (SpO2) during sleep in those infants.

METHODS

The study is a retrospective, descriptive case series of infants attending PWS clinic at Mater Children’s Hospital, Brisbane (MCH). Infants had polysomnography (PSG) in a fully equipped sleep laboratory (MCH). Electroencephalography (EEG), electrooculography (EOG), electromyography (EMG) (Network Concepts, Inc), respiratory inductance plethysmography of ribcage and abdominal wall (Respitrace), oxygen saturation (Ninametrics), transcutaneous carbon dioxide measurement (Radiometer Copenhagen) and nasal airflow by a pressure transducer (Sullivan) were recorded. PSG studies were analyzed according to the 2007 AASM guidelines for sleep scoring.12 and central apnoea was defined as the absence of airflow for >3 second. Central hypopnoea was scored when a 50% reduction in airflow with reduced respiratory effort was
The primary outcomes assessed were central apnoea index (CAI), central event index (CEI), obstructive event indices, duration of central apnoea, % sleep time below 90% saturation and number of desaturation episodes below 90%. Central event indices during rapid-eye movement (REM) and non-REM sleep were analysed in a subset of subjects.

All children reported in this study had episodes of desaturations associated with central events, and were started on supplemental oxygen during sleep. Each child was followed at 3-monthly intervals with split-night studies (periods in both air and supplemental oxygen). For the majority (22/30) of studies, the study was commenced in room air and oxygen titrated after at least one sleep cycle was obtained. In a minority (8/30) of studies, the study began in oxygen with subsequent time in room air. A database of results was compiled using Microsoft Excel, and statistical analyses were performed using SPSS for Windows V15.0. Non-parametric (Wilcoxon signed rank) tests were used to compare data in air and oxygen arms for our subject cohort. Ethics approval was obtained from the hospital ethics committee.

RESULTS

Thirty split-night studies on 10 infants (8 male) aged 0.06–1.79 years with a median (IQR) age of 0.68 (0.45, 1.07) years were undertaken. The baseline characteristics of the study population are shown (table 1). A median (IQR) total sleep time of 501 (448, 527) min was measured across the group.

At baseline (ie, while in room air), children with PWS had a median (IQR) CAI of 4.7 (1.9, 16.7) per hour, with accompanying falls in SpO2. Oxygen therapy led to statistically significant reductions in CAI to 2.5 (0.9, 5.6) per hour (p=0.002) (figure 1) as well as improved SpO2 (table 2). The median CEI was also higher in air (10.4, 18.9) compared with 5.3 (2.4, 9.5) in oxygen (p<0.001). No change in the number of obstructive events was noted. No differences in sleep efficiency were seen between the air and oxygen arms of the study.

The association of events with sleep stage was investigated and these data are shown in table 3. Sleep stages were dichotomized as 1 REM sleep or active sleep (AS) and 2 non-REM or quiet sleep (QS). The median (IQR) central apnoea (apnoea and hypopnoea) index (CAI) was noted in air to be 15.9 (8.6, 32.8) events h⁻¹ during REM/AS and 4.4 (3.5, 7.9) during non-REM (p<0.001). In oxygen at 250 ml/min the CAI was 9.1 (4.2, 18.6) during REM/AS and 1.9 (0.4, 4.3) during non-REM (p<0.001). Thus events were more prominent in REM sleep in both air and oxygen arms. Significant falls in CEI from baseline (air) during REM (p=0.001) and non-REM sleep (p=0.002) were noted with oxygen therapy (see table 3).

A trend towards improvement over time was noted. Of the 10 infants studied, seven had three or more split-night studies
undertaken over the study duration. Study 1 was undertaken at a median (IQ) age of 0.42 (0.12, 0.63) years, study 2 at 0.67 (0.39, 0.88) years and study 3 at 0.94 (0.68, 1.32) years. The median (range) duration between study 1 and study 2 was 13 (12–15) weeks, and was 14 (9–14) weeks between study 2 and study 3. The evolution with age and improvement in central apnoea and central event frequency over the course of studies 1–3 for this group of seven patients are illustrated in figure 2.

DISCUSSION

Respiratory control during sleep develops rapidly through infancy and may be integral to the neurodevelopment maturation that is taking place in such babies. There is evidence in the literature for the presence of central apnoea and dysrhythmic breathing in normal infants, which progressively improves with age,16,17 and also evidence of abnormal respiratory control in PWS from both human and rodent studies.5–14 The mechanisms by which such central events occur are hypothesised to be related to abnormalities in respiratory control allowing the crossing of a threshold by supraspinal and spinal mechanisms. It is thought that a stimulus (e.g., hypoxia, arousal) results in a transient increase in ventilatory drive. In a normal individual the increased ventilation would continue until the resultant reduction in paCO2 is detected at the chemoreceptors, but for reasons of poor respiratory control (e.g., PWS), this mechanism overshoots and results in a fall in paCO2 to below the supraspinal level and the reaching of ‘apnoea threshold’4. A central apnoea thus occurs, which may result in desaturation or be terminated by an arousal, both stimuli that may further perpetuate such a cycle of events by increasing drive.18 It is known that the NDN (necamin) gene is deleted in children with PWS, and in a murine model, knock out of the NDN gene led to abnormal development of the pre-Bötzinger complex and a respiratory phenotype with abnormal breathing and prolonged central apnoea.19 Furthermore, PWS patients are reported to have blunted responses to hypoxia and hypercapnia and abnormal chemoreceptor sensitivity.11–14 PWS infants appeared to have greater respiratory stability in oxygen, and the mechanism would support this, for if by eliminating hypoxia as a causal factor in the cascade by which central events are generated, one might expect event frequency to be markedly reduced. Figure 3 highlights the purported mechanism by which central apnoea arise in children with PWS and blunted chemoreceptor sensitivity.

In our group of infants, reduced respiratory effort was noted in REM and non-REM sleep stages, although was more marked in REM/AS, with increases in central event numbers as well as increased number of falls in SpO2. This was witnessed in both air and oxygen arms of the study and one limitation of our study is the trend towards a shorter duration of REM/AS that was captured in oxygen versus air. This is likely to reflect the fact that functional residual capacity (FRC) of the lung is lower in REM sleep, and SpO2 dips are thus more likely to result from apnoea events, reinforcing the cyclical mechanism for respiratory control.

Oxygen Therapy

Hypoxia

Increase ventilation

Arcal

Immature respiratory control allows overshooting and marked (paCO2

Immature respiratory control leads to fall to well below eupnic paCO2

FIGURE 3 Proposed mechanism by which abnormalities in respiratory control result in central apnoea in children with PWS syndrome.
aberrant respiratory control highlighted in Figure 3. As infants grow older the degree of desaturation lessened, while at the same time the rhythmic breathing pattern steadily improved (Figure 2a, b) which may support changes in FRC as a significant contributory factor to such events. With advanced age, and a greater FRC (due to steady improvements in muscle tone, coupled with lung growth and changes in lung mechanics), desaturation is less likely to occur in association with a short period of apnoea, and thus the substrate of hypoxia required to perpetuate events is no longer present.

In our case series, we report the presence of central apnoea in REM sleep, which may share similarities with the REM hypventilation reported in older children with PWS who are obese.20 21 Thus, a dyssynchronous breathing pattern seen in PWS may be inherent to the condition (due to the chemoreceptor insensitivity that arises from the NDN gene deletion) and which can express itself in the presence of appropriate factors, namely hypoxia and immature respiratory control in infancy, or obesity in the older child.

CONCLUSIONS

Infants with PWS have sleep-disordered breathing problems, which are predominantly central in origin, and cause significant hypoxia in some patients. Improvements in both central event indices and oxygenation were noted on oxygen therapy. Longitudinal work with this patient group would help to establish the timing of onset of obstructive symptoms. Whether early recognition of central hypoventilation and correction with oxygen after the evolution of respiratory dysfunction in this patient group remain to be seen.

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