

# Impact of genetic background and molecular mechanisms on Prader-Willi syndrome phenotype

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**UNIVERSITY OF ZAGREB**

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**Impact of Genetic Background and Molecular  
Mechanisms on Prader-Willi Syndrome  
Phenotype**

**Graduate thesis**



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This graduate thesis was made at the Department of Paediatrics,  
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## Abbreviation

ASD	Autism Spectrum Disorder
DMR	Differentially Methylated Regions
FISH	Fluorescence <i>in situ</i> hybridization
GH	Growth Hormone
lncRNA	Long Non-Coding RNA
MS-HRM	Methylation-Sensitive High-Resolution Melting
MS-MLPA	Methylation Specific Multiplex Ligation-Dependent Probe Amplification
MS-PCR	Methylation Specific Polymerase Chain Reaction
mUPD	Maternal Uniparental Disomy
PWS	Prader-Willi Syndrome
SNP	Small Nucleotide Polymorphisms
snoRNA	Small Nucleolar RNAs

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## 1.0 Abstract

**Title:** Impact of Genetic Background and Molecular Mechanisms on Prader-Willi Syndrome Phenotype.

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Prader-Willi syndrome (PWS) is a complex disorder with a vast variable clinical presentation, resulting from the loss of paternally inherited genes on chromosome 15q11-q13. The loss of expression of one or more genes located within the PWS critical region results in the various phenotypes. PWS can occur via three different distinct genetic mechanisms including paternal deletion of the 15q11-q13, maternal uniparental disomy (mUPD), or imprinting defects. Although there are some clinical symptoms that are shared among PWS patients, the overall complexity of the syndrome results in unique clinical presentations, emphasizing the importance of genetic diagnosis and counseling, where individual based therapy can be implemented. DNA methylation status was first confirmed, followed by the identification of one of the three molecular mechanisms. Individuals who suffered from paternal deletions shared common symptoms like low birth weight, lower IQ, hypopigmentation and maladaptive behaviours. The type of PWS deletion (type I or II) also impacts the clinical presentation, with type I patients having a more severe presentation and acquiring speech later than those with type II. Individuals with mUPD tend to present with lower birth length, various sleep disorders, psychosis and Autism Spectrum Disorder (ASD). In addition, the average maternal age in the mUPD patients was higher than those with deletions. No single gene mutation has been shown to contribute to PWS or related to any exclusive symptoms. Although some genes like MKRN3, MAGEL2, NDN do not address the full spectrum of symptoms, others have noted that SNORD116 located within the SNURF-SNRPN complex has become a critical candidate for PWS due to its role in growth retardation and neuroendocrine disturbances leading to hyperphagia and obesity. The broad spectrum of PWS observed in patients and the absence of a clear genotype-phenotype relationship, implies that various genes involved, have an intensified effect on the various phenotypes when lost.

**Keywords:** Prader-Willi syndrome, Genotype, Phenotype, Maternal uniparental disomy

## 2.0 Sažetak

**Naslov:** Utjecaj Genotipa I Molekularnih Mehanizama Na Fenotip Prader-Willijevog Sindroma

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Prader-Willijev sindrom (PWS) je složen poremećaj s promjenjivom kliničkom slikom, koji nastaje kao posljedica gubitka funkcije očevih gena na kromosomu 15q11-q13. Gubitak ekspresije jednog ili više gena smještenih unutar PWS kritične regije rezultira pojavom različitih fenotipova. PWS se može pojaviti putem tri različita genetska mehanizma, uključujući deleciju očevih gena u određenoj regiji kromosoma 15q11-q13, majčinsku uniparentalnu disomiju (mUPD) ili defekt genomskog upisa. Iako postoje neki klinički simptomi koji su jednaki među pacijentima sa PWS-om ukupna složenost sindroma rezultira jedinstvenom kliničkom slikom naglašavajući važnost genetske dijagnoze i savjetovanja, nakon kojih se može provesti individualna terapija. Prvo se potvrđuje status metilacije DNA, a zatim slijedi identifikacija jednog od tri molekularna mehanizma. Pojedinci koji pate od delecije DNA oca dijele uobičajene simptome poput niske tjelesne težine, nižeg IQ-a, hipopigmentacije i neprilagođenog ponašanja. Tip delecije (tip I ili II) također utječe na kliničku prezentaciju, gdje pacijenti s tipom I imaju jače izraženu kliničku sliku i kasnije usvajaju govor od onih s tipom II. Pojedinci s mUPD-om pretežito imaju manju porođajnu dužinu, razne poremećaje spavanja, psihozu i poremećaje iz spektra autizma (ASD). Prosječna dob majke u mUPD bolesnika je viša od one s delecijama. Dokazano je da nijedna specifična mutacija ne uzrokuje PWS ili je povezana s bilo kojim isključivim simptomima. Iako se neki geni poput MKRN3, MAGEL2, NDN ne bave čitavim spektrom simptoma, primjećeno da je SNORD116 smješten u kompleksu SNURF-SNRPN postao kritični kandidat za PWS zbog njegove uloge u usporavanju rasta i poremećaja neuroendokrinog poremećaja koji dovode do hiperfagije i pretilosti. Široki spektar simptoma koji se nalazi u PWS-u i odsutnost jasnog odnosa genotip-fenotip podrazumijeva da različiti geni, kada nisu izraženi, imaju akumulativni učinak na različite fenotipove.

**Ključne riječi:** Prader-Willijev sindrom, Genotip, Fenotip, Majčinska uniparentalna disomija

### 3.0 Introduction

Prader-Willi Syndrome (PWS) is a relatively rare (~1/10 000-30 000) complex genetic condition which has the potential to involve a variety of different systems within the human body.<sup>1</sup> The PWS critical region, 15q11-q13, located on chromosome 15 is monoallelically expressed exclusively through paternally inherited genes. The loss of expression of one or more of these genes contributes to the variety of phenotypes one can expect in a patient with PWS.<sup>2</sup> The absence of gene expression occurs through a variety of different mechanisms, further increasing the variability of the phenotypic outcome. Due to the complexity and variability of the inheritance patterns and the variety of genes that may be affected, the outcome of the disorder needs to be examined on an individual basis.

Clinical manifestations vary from patient to patient and become apparent at different stages of life.<sup>3</sup> Prenatally, the growth parameters of the fetus are usually within the normal range. However, compared to unaffected siblings, the birth weight and BMI is 15% lower on average. Hypotonia may also occur during this time, presenting as decreased fetal movements and abnormal fetal position.<sup>4,5</sup>

During infancy, severe hypotonia is a clinical hallmark of PWS, leading to failure to thrive as a result of lethargy and poor sucking.<sup>6</sup> By 9 months of age, these eating behaviours begin to normalize, and the hypotonic status starts to improve. However, some hypotonia persists throughout life and thus results in reduced muscle mass and tone. Also, during this period, characteristic behavioural problems are common, such as stubbornness, compulsiveness, and self-injurious behaviour.<sup>7</sup>

Social and physical milestones such as reading, first words, sitting and walking are delayed by up to double the normal age. Many children have mild learning difficulties, intellectual disability and display poor academic performance.<sup>8</sup> Another common feature in

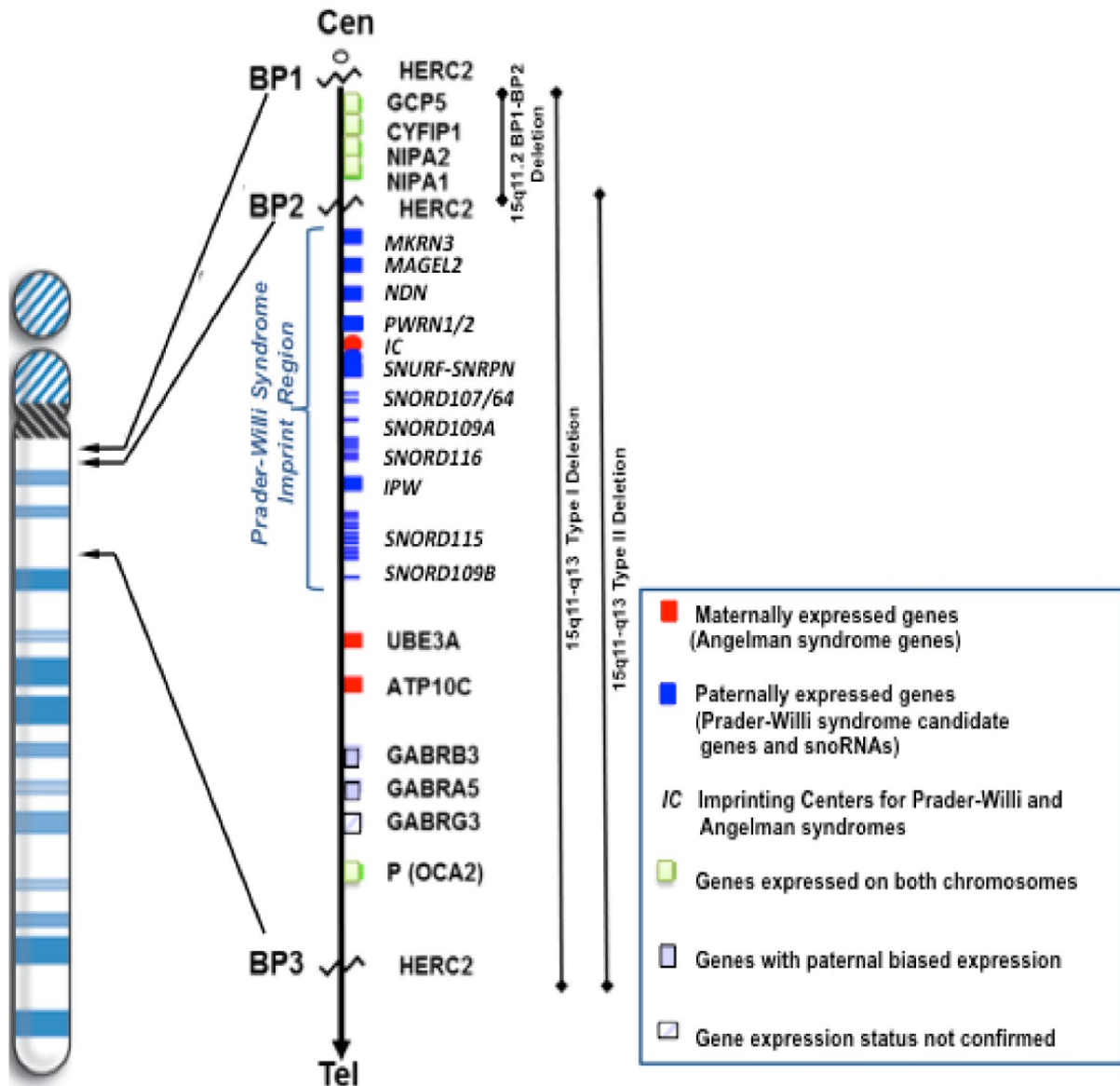


this syndrome is sleep apnea which results in impaired quality of sleep. Sleep disturbances such as this are frequently associated with daytime sleepiness and sedentary behaviour.<sup>9</sup>

During childhood, individuals suffer from severe obesity, unless food intake is strictly controlled by the family. Hyperphagia and obsessive food seeking is due to hypothalamic dysfunction, resulting in a lack of satiety.<sup>10</sup> Individuals with an uncontrolled condition usually suffer from cardiovascular problems, respiratory insufficiency, metabolic syndrome, sleep apnea and diabetes mellitus type 2.<sup>11</sup> These complications are the major causes of morbidity and mortality among these individuals, ranging from 1.25-3% per year.<sup>12</sup>

The most affected system in PWS is the endocrine system, likely due to hypothalamic dysfunction. The presence of growth hormone (GH) deficiency (74%) is associated with short stature and small hands and feet.<sup>13</sup> Other endocrine manifestations include hypogonadism among both sexes and is expressed as hypogonadism, incomplete pubertal development and infertility in most individuals.<sup>14</sup> Other endocrine disturbances include hypothyroidism (20-30%), central adrenal insufficiency (5%) and type 2 diabetes mellitus (25%) due to obesity complications.<sup>15,16</sup>

Due to the vast variety of clinical symptoms an individual with PWS can expect, it is important to identify the mechanism of inheritance and the specific gene(s) affected in order to help anticipate and target the expected clinical presentation as early as possible.



**Figure 1.** Chromosome 15 ideogram and locations of breakpoints (BP1, BP2 and BP3) and imprinted and nonimprinted genes between the breakpoints in the 15q11.2-q13.1 region.<sup>17</sup>

#### 4.0 Molecular mechanisms and diagnostics related to Prader-Willi Syndrome

There are three distinct genetic mechanisms in which PWS may develop in an individual. Each mechanism is due to a common genetic discrepancy that eliminates the

expression of the imprinted paternal genes.<sup>18</sup> Majority of PWS patients (65-75%) present with a 5- 6 Mb paternal deletion within the 15q11-q13 region. In 95% of patients with a paternal deletion, two major types have been defined.<sup>19,20</sup> Type 1 patients are described to have breakpoints BP1 (proximal) and BP3 (distal), while those of type II patients have a different proximal breakpoint (BP2) while sharing the same distal breakpoint, BP3, as type 1 patients. (Figure 1) In the remaining 5%, different distal breakpoints have been identified, however, not much is known. For approximately 20-30% of cases, maternal uniparental disomy (mUPD) occurs.<sup>17</sup> This transpires when both copies of chromosome 15 are inherited from the mother, resulting in the absence of paternal genes. Imprinting defects account for 1-3% of PWS cases and are caused by epimutations or microdeletions in the PWS imprinting center.<sup>21</sup>

The complexity of the molecular and clinical aspects of PWS emphasizes the importance of genetic diagnosis and counseling, in order to provide effective and efficient individual-based therapy. DNA methylation analysis can correctly diagnosis PWS consistently in all three assays and differentiate it from Angelman syndrome in 99% of cases.<sup>2</sup> Currently there are three different assays with this detection capacity: the gold standard is methylation-specific PCR (MS-PCR), methylation specific multiplex ligation-dependent probe amplification (MS-MLPA) and methylation-sensitive high-resolution melting (MS-HRM).<sup>22,23</sup> This targets the 5' CpG island of the SNURF-SNRPN locus, central to the PWS imprinting center, and results in the detection of the unmethylated paternal allele being expressed and the methylated maternal allele being repressed.<sup>24</sup>

Once the methylation status is confirmed, distinguishing between the molecular mechanisms are important for genetic counseling. Risk of recurrence for sporadic deletions is very low (<1%), however, cases involving structural abnormalities (for example translocation or inversion) involving chromosome 15 can be as high as 25-50%. Fluorescence in situ

hybridization (FISH) is used in order to detect the source of the deletion.<sup>25,26</sup> mUPD is generally de novo with a recurrence rate of <1%. However, the patient and parents should undergo investigations by small nucleotide polymorphisms (SNP) microarray for appropriate genetic counseling.<sup>27</sup> Most imprinting defects are caused by epimutations without alterations in the DNA sequence and have <1% recurrence risk. Unfortunately, 15% of individuals with imprinting defects express paternally inherited microdeletions within the PWS imprinting center, resulting in a significant 50% recurrence risk. These individuals should undergo imprinting center analysis by MS-MLPA or DNA sequencing in order to locate the origin of the defect.<sup>28</sup>

## 5.0 Genotype-phenotype relationships in Prader-Willi Syndrome

### 5.1 Comparison of clinical presentation among PWS deletion patients with different major subtypes

The genetic changes found in patients with PWS are not associated with exclusive symptoms. Nevertheless, there has been great effort to show the differences in frequency and severity in the clinical presentation across the various mechanisms of inheritance (deletion, mUPD, imprinting errors). All individuals who suffered from paternal deletions experienced a classical phenotypic presentation including hypotonia, feeding disorders, developmental delay and learning disorders.<sup>18</sup> However, as shown by Butler et al., the type of deletion (type I or II), also impacts the clinical presentation. It was found that individuals with a type I deletions had a more severe presentation when compared to type II individuals. These patients demonstrate greater self-injurious behaviour, deficit in adaptive skills, obsessive compulsive behaviour and learning difficulties. It is also worth mentioning that, patients with

type I deletions, tend to acquire speech later than those with type II (4.3years old vs 3.4 years old).<sup>29</sup>

When comparing the two major types of deletions (I vs II), four genes (NIPA1, NIPA2, CYF1P1 and GCP5) have been identified between the proximal breakpoints BP1 and BP2.<sup>30</sup> Although all four genes appeared to contribute to some degree, NIPA2 seemed to have the most significant impact.<sup>31</sup> Bittle et al. demonstrated that NIPA1, NIPA2 and CYF1P1 may influence the behavioural and cognitive parameters, as well as, play an important role in central nervous system development and function.<sup>32</sup>

## 5.2 Comparison of clinical presentation among various mechanisms of inheritance

For comparison, patients were classified as either deleted or non-deleted. Individuals with deletions were more likely to experience low birth weight, feeding difficulties, sleeping disturbances, hypogonadism and speech and language deficits when compared to non-deleted individuals.<sup>33</sup> Spritz et al. illustrated that deleted patients are more likely to experience hypopigmentation due to the loss of P gene, which is associated with OCA2, the most frequent form of tyrosinase-positive oculocutaneous albinism.<sup>34</sup> Individuals with UPD were 31.6 times more likely to have a higher verbal IQ than their performance IQ when compared with those with UPD.<sup>35</sup>

Controlling for the higher IQ in non-deleted patients, Dykens et al. verified that deleted patients showed significantly higher cases of maladaptive behaviour in the form of obsessive-compulsive behaviour, skin picking, nail biting, sulking and withdrawing.<sup>36</sup> Individuals with deletions presented at a higher frequency with seizures than those non-deleted patients.<sup>18</sup> It has been proposed that the increased prevalence of seizures in deleted individuals is due to the loss of seizure related genes (such as GABRB3) in the region.

Deleted patients were also more prone to febrile seizures, suggesting defects in the thermoregulatory centre.<sup>37</sup>

Numerous other characteristics are more prevalent in mUPD patients, a group of individuals associated with advanced maternal age. These individuals are more likely to experience a lower birth length, sleeping disorders, psychosis and autism spectrum disorder.<sup>29,36,38</sup> Williams et al. reported that all mUPD patients in the study had some form of sleep disordered breathing (SDB), however, the type and severity of SDB was not predicable based on the underlying genetic defect. Sleep disorders observed varied from obstructive sleep apnea, abnormal arousal, insomnia, abnormal circadian rhythm during REM sleep and excessive daytime sleepiness.<sup>39</sup> Boer et al. hypothesized that psychosis and autism spectrum disorder may be related to the inheritance of two maternal copies. Novel differentially methylated regions (DMR) have been identified within the PWS critical region, involving an increased expression of maternally imprinted genes. These genes have receptors for the brains primary inhibitory neurotransmitter which may be involved in the development of psychosis in mUPD individuals.<sup>40</sup> Varela et al. reported a higher verbal IQ, milder behavioural disturbances, earlier age of walking, less likely to develop characteristic facial features and hypopigmentation when compared to deleted patients.

**Table 1.** Prevalent clinical symptoms in PWS patients with deletions and mUPD.

<b>Individuals with deletions</b>	<b>Individuals with mUPD</b>
Low birth weight	Lower birth length
Feeding difficulties	Sleep disorders
Speech and language deficit – Lower IQ	Psychosis
Hypogonadism	Autism Spectrum Disorder (ASD)
Hypopigmentation	
Maladaptive behaviours	
Seizures	

### 5.3 Comparison of clinical presentation among various gene disturbances within the PWS critical region

No single gene mutation has been shown to contribute to PWS. Each gene within the PWS critical region is independently examined in order to demonstrate the clinical phenotype related to the syndrome. The Makorin Ring Finger Protein 3 (MKRN3) gene is paternally expressed throughout adult human tissue with highest concentration in the testis. MKRN3 is associated with the hindrance of the initiation of puberty, and loss of function mutations of the gene have been reported as the main cause of central precocious puberty.<sup>41</sup> Several authors demonstrated the association between the MKRN3 gene and puberty dysfunction through the use of experimental mice models. This signified a possible role in the hypothalamic-pituitary-gonadal axis potentially leading to the clinical presentation of hypogonadism and infertility in PWS.<sup>42,43</sup>

The clinical presentation one can expect from the loss of expression of MAGEL2 gene has been shown to cause endocrine dysfunction, similar to individuals with PWS. Bischof et al. displayed this presentation as neonatal growth retardation, weight gain, increase adiposity, impaired hypothalamic regulation and changes to the circadian rhythm.<sup>44</sup> Commonly observed among children with PWS is hyperphagia, a condition connected with a defect in the hypothalamic-arcuate nucleus. This is the major action site for many interactions between neuropeptide Y (NPY), agouti-related peptide (AgRP), proopiomelanocortin (POMC) and leptin, in response to regulating food intake and managing body weight.<sup>45</sup> A loss of MAGEL2 expression disrupts leptin-mediated depolarization of POMC neurons, resulting in minimal repression of food intake and uncontrolled fat storage. Varela et al. reported loss of MAGEL2 gene also impairs reproductive function in mice, through prolonged and irregular estrous cycles in females and decreased testosterone levels in males

suggesting some contribution to the reproductive deficiencies observed in some patients with PWS.<sup>46</sup>

The *Necdin* (*NDN*) gene is highly expressed in mature hypothalamic neurons and has been proposed to be a key regulator of GnRH levels, modifying essential intracellular processes.<sup>47</sup> Loss of *NDN* expression, results in a decrease number of GnRH neurons leading to complications in hypothalamus during development, which may contribute to the hypogonadism and infertility seen in PWS.<sup>48</sup> Through experimental studies, it was observed that *NDN* paternal-deficient mice were associated with changes in serotonin and respiratory systems, presenting as irregular breathing and sleep apnea, which are features displayed in PWS. Zanella et al. demonstrated the importance of the *NDN* gene, when compared to *NDN*-KO mice, where they suffered sudden death, due to respiratory disorders, proposing that *NDN* may be a genetic factor contributing to respiratory dysfunctions and apneas in PWS.<sup>49</sup>

Kanber et al. discovered a few patients with abnormal deletions related to PWS. The first patient has a deletion of *MKRN3*, *MAGEL2* and *NDN* however, experienced no major criteria for PWS except for obesity, developmental delay and high pain threshold. Patient 2 and 3 had a deletion accompanying several other genes (*NPAP1*, *SNURF-SNRPN* and *SNORD* genes) but not reaching *MKRN3*, *MAGEL2* and *NDN* and presented with major clinical signs.<sup>50</sup> The study suggested that the paternal deficiency of these three genes alone was not sufficient to cause PWS presentation and proposed *NPAP1*, *SNURF-SNRPN* and the *SNORD* genes are located within the critical region of PWS.

The critical region of PWS contains a series of long non-coding RNAs (*lncRNAs*), which can potentially to be involved in epigenetic modifications of DNA and the regulation of gene expression.<sup>51</sup> Prader-Willi Region Non-Protein Coding RNA 1 (*PWRN1*) is the first *lncRNA* in the PWS critical region and was found to be an alternative 5' part of the *SNURF-SNRPN*.<sup>52</sup> The Nuclear Pore Associated Protein 1 (*NPAP1*) is an intronless gene that is



monoallelically expressed in the fetal brain, including the hypothalamus, possibly explaining for various endocrine disturbances individuals with PWS experience.<sup>53</sup> NPAP1 is associated with the Nuclear Pore Complex (NPC), where the main function is to regulate the transport of macromolecules between the nucleus and cytoplasm. There are also some nuclear processes in which the NPC is involved with, including gene regulation, cell cycle control and the generation mRNA.<sup>54</sup> However, due to the lack of gene orthology in mice, the exact role of PWRN1 and NPAP1 in the development of PWS is still unclear.

The Small Nuclear Ribocleoptien Polypeptide N (SNRPN) gene is located within the critical region of PWS and is essential for its regulatory role over the imprinted gene within chromosome 15.<sup>55</sup> The SNRPN Upsteam Reading Frame (SNURF) gene is encoded by an evolutionarily conserved upstream open reading frame and found within the nucleus. Together SNURF-SNRPN is a complex bicistronic gene, encoding for two different proteins while containing the PWS imprinting centre at its 5' end. SNURF produces a small nuclear protein of unknown function while the SNRPN segment encodes for SmN, involved in mRNA splicing. It also holds six Small Nucleolar RNA (snoRNA) genes located telomerically and will be discussed individually.<sup>56</sup>

The series of snoRNAs located within the SNURF-SNRPN complex are thought to participate in DNA methylation, alternative splicing and post-transcriptional regulation.<sup>57</sup> There are five single copy genes (SNORD64, SNORD107, SNORD108, SNORD109A and SNORD109B) located within the PWS region and two gene clusters (SNORD115 and SNORD116). The present understanding of the single copy snoRNAs is limited however, some advancements in the understanding of the snoRNA gene clusters have been made. These sequences are conserved across placental mammals, suggesting having an evolutionary role<sup>58</sup>. Ding et al. demonstrated the importance of the SNORD116 cluster and PWS phenotype through an experimental study on mice. They reported the snord116 knockout

mice displayed typical PWS features such growth retardation, and hyperphagia due to the important role of controlling NYP neuronal functions and ultimately food consumption.<sup>59</sup> Burnett et al. also discovered that mice deficient in SNORD116, also had decreased activity of the prohormone convertase, impairing the prohormone processing of proinsulin, pro-GH-releasing hormone and proghrelin contributing to the main neuroendocrine features in PWS.<sup>60</sup> Currently, SNORD116 has emerged as a critical candidate for PWS not only because it is located within the PWS critical region, but also because paternal deletions of the previously discussed genes, do not address the full clinical presentation of PWS.

The imprinted in Prader-Willi Syndrome (IPW) gene is expressed among both fetal and adult tissues, exclusively from the paternal allele. It was proposed that the lack of IPW gene causes abnormal upregulation of maternally expressed genes, which was supported by clinical reports of mUPD patients presenting with a typical clinical presentation including: neonatal hypotonia, small hands and feet, hyperphagia and intellectual disability.<sup>61</sup>

SNORD115 gene presents a complementary sequence with the mRNA encoding the serotonin receptor 5-HT<sub>2C</sub>.<sup>57</sup> Ding et al. demonstrated that large deletions including SNORD115 developed normally to adulthood, implying that the lack of SNORD115 alone, is not sufficient to cause PWS.<sup>62</sup> Nonogaki et al. performed an experimental study which described serotonin knockout mice to have developed hyperphagia and late onset obesity, two major PWS phenotypes. This shows that when absent along with other genes in the PWS critical region, the patient cannot be excluded for PWS.<sup>63</sup>

## 6.0 Conclusion

Imprinting disorders like PWS, are complex processes produced through the lack of expression of paternally inherited genes on chromosome 15. PWS varies on an individual basis due to the numerous clinical presentations and various systems involved, including

neurological, endocrine and metabolic. These variants are possible due to multiple methods of inheriting the syndrome and numerous genes involved. The mechanisms of inheritance (deletion, mUPD and imprinting defects) were compared and the prevalence of symptoms were determined for each. Also, the PWS critical region contains many genes involved in the disorder, however, no single gene mutation has been shown to produce PWS. For these reasons, it is important to identify the method of inheritance and genes involved with each patient, in order to identify the potential clinical presentation, one may expect and develop a therapeutic plan and/or genetic counselling for the different levels of severity.

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## 9.0 Biography

Patrick Michael Galvano was born on May 25<sup>th</sup>, 1990 in Windsor Ontario Canada. After completing high school in 2008, Patrick was accepted in the biological science programme at the University of Windsor, where he completed a four-year undergraduate degree, focusing on environmental studies. In the final year of his undergraduate degree, he successfully completed and defended his undergraduate thesis, which was soon accepted into the journal of reproductive biology in 2013. Upon graduating, he was employed by Dr Jan Ciborowski at the University of Windsor as a research assistant where he was a part of several projects including the coastal wetland monitoring project and great lakes environmental indicator project. In September of 2013, Patrick embarked on his medical journey in Zagreb, Croatia at the University of Zagreb, School of Medicine. Over the course of his studies, he has developed a passion for paediatrics and primary care.