Analysis of the evolving clinical phenotype and molecular mechanisms in Williams-Beuren syndrome

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"Let me tell you the secret that has led me to my goal. My strength lies solely in my tenacity".

Louis Pasteur

A mis papis,
a Andy, a Titi,
y a Mauri.

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ABSTRACT

Williams-Beuren Syndrome (WBS, MIM 194050) is a neurodevelopmental disorder with multisystemic manifestations of variable expressivity, including dysmorphic features, vascular stenoses, and intellectual disability with an uneven neurocognitive profile. It is caused by a recurrent 1.55-1.83 Mb deletion secondary to non-allelic homologous recombination (NAHR) at chromosome band 7q11.23 that includes 26 to 28 genes. A genotype-phenotype correlation has only been established for some of the genes involved in this deletion, the most studied is the elastin gene associated with the cardiovascular phenotype. In this thesis project, we have aimed to further elucidate the molecular mechanisms involved in NAHR, as well as possible *cis* and *trans*-acting mechanisms. We have also described novel clinical characteristics (metabolic phenotype), described in greater detail the cardiovascular phenotype present in patients with WBS, as well as determining possible candidate genes associated with both of these phenotypes.

RESUMEN

El síndrome de Williams-Beuren (SWB, MIM 194050) es un trastorno del neurodesarrollo que cursa con manifestaciones multisistémicas de expresividad variable, incluyendo rasgos dismórficos, estenosis vasculares, discapacidad intelectual con un perfil neurocognitivo desigual. Está causado por una deleción de 1.55-1.83 Mb secundario a una recombinación homóloga no alélica (NAHR) en la banda cromosómica 7q11.23 que incluye entre 26 a 28 genes. Una correlación genotipo-fenotipo ha sido establecida para muy pocos genes dentro de la región, el más estudiado ha sido el gen de la elastina relacionado con el fenotipo cardiovascular. En este proyecto de tesis, hemos incrementado el conocimiento acerca de los mecanismos moleculares involucrados en la NAHR, incluyendo posibles mecanismos que pueden interaccionar en *cis* o en *trans*. También hemos descrito nuevas y previamente reportadas manifestaciones

clínicas del fenotipo metabólico y hemos descrito en mayor detalle el fenotipo cardiovascular, hemos buscado posibles genes candidatos asociados a ambos fenotipos.

PROLOGUE

Numerous researchers have studied Williams-Beuren syndrome since it was first described in the 1960's with the objectives of determining the phenotype and the natural history of the syndrome, elucidating the causal molecular mechanisms involved and the genotype-phenotype correlations. The final objective of the effort of the scientific community is to improve the medical management of patients, by establishing follow-up guidelines, medical treatment and therapies to improve their physical and psychological symptomatology.

The global aims of this thesis project are to improve the definition of the WBS phenotype, the natural history of this disorder, to characterize the deletion and the molecular mechanisms involved, establish clinical-molecular correlations to identify possible candidate genes associated with specific phenotypes.

The **introduction** focuses on the clinical characteristics and management of the WBS, an overview of the molecular mechanisms involved and previously reported chromosomal rearrangements, and previously known genotype-phenotype correlations.

The **thesis body** is divided in three chapters that present in detail the methodology used and the results obtained. The **first chapter** is about the molecular mechanisms involved in WBS. The **second chapter** describes the metabolic phenotype. The **third chapter** describes cardiovascular disease and hypertension in WBS.

The global **discussion** aims to integrate the results obtained and compare them to previous knowledge.

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INTRODUCTION

1. WILLIAMS BEUREN SYNDROME CLINICAL CHARACTERISTICS

Williams Beuren syndrome (WBS, OMIM 194050) is a neurocognitive disorder characterized by multisystemic manifestations of variable expressivity. It has an incidence of 1/7,500 – 1/10,000 individuals [1], almost always with a sporadic occurrence, although it can be transmitted in an autosomal dominant manner [2].

WBS was first described in 1952 in a report encompassing the clinical characteristics of children who presented infantile hypercalcemia, short stature and congenital malformations [3]. It was several years later that Dr. John Cyprian Phipps Williams and Dr. Alois Beuren described the main characteristics of this disorder; supravalvular aortic stenosis, intellectual deficit and common facial characteristics [4, 5].

1.1 Facial Phenotype

The facial gestalt of WBS is unique and distinct from other neurodevelopmental disorders. In infants and young children it is characterized by a broad forehead, bitemporal narrowing, low nasal root, bulbous nasal tip, strabismus, an iris stellate pattern, periorbital fullness, malar flattening, long philtrum, wide mouth, full lips, full cheeks and widely spaced teeth with malocclusion [6] (Figure 1.A).

Older children and adults usually have a more gaunt appearance of the face that is characterized by prominent supraorbital ridges, a narrow nasal root, full nasal tip, malar flattening, wide mouth and full lips, small jaw, dental malocclusion and long neck with sloping shoulders [6] (Figure 1.B and 1.C).

Figure 1. Typical fancies of three unrelated patients with WBS. A. The young child has a flat nose bridge, upturned tip of the nose, long philtrum and full cheeks. **B.** The school-aged child has full lips, wide mouth and mildly spaced teeth. **C.** The young adult has a prominent nose and nasal tip, wide mouth and a full lower lip. From [16].

1.2 Growth and Puberty

The incidence of prenatal growth deficiency is approximately 50-70% [7, 8]. During the first two years of age WBS individuals usually have poor growth and follow the 3rd percentile curve until 9 and 11 years of age in females and males, respectively[8]. The rate of linear growth during childhood is 75% of normal [9]. WBS individuals present a premature and brief pubertal growth spurt. Menarche usually occurs at an earlier age [8]. Mean adult height is at the 3rd percentile or lower for 70% of WBS individuals [8].

Several reports have shown that between 25 to 65% of adults with WBS have a body mass index (BMI) compatible with an overweight to obesity range [10, 11].

1.3 Cardiovascular Phenotype

One of the main hallmarks of WBS phenotype is cardiovascular (CV) disease, which is characterized by stenoses of medium and large arteries [12]. The reduction of elastin content in the media of arteries

may lead to recurrent injury and fibrosis; as well as an increase in hemodynamic stress to the vascular endothelium, which ultimately leads to proliferation of smooth muscle cells and fibroblasts, fibrosis and a subsequent intimal hyperplasia [13–15]. Vascular stenoses can occur in any artery of the body; however the most frequent locations are the supravalvular aortic (Figure 2) and peripheral pulmonic stenosis [12].

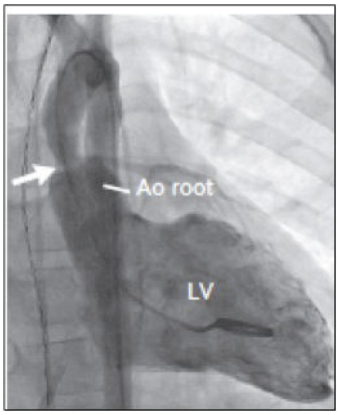

Figure 2. Left ventriculography shows severe supravalvular aortic stenosis (arrow). A dilatation of the aortic root with mild hypoplasia of the proximal ascending aorta is observed. From [16]

Supravalvular aortic stenosis (SVAS) occurs in approximately 70% of WBS individuals, with a severity that ranges from mild to severe [16–18]. This cardiovascular involvement tends to progress with time, especially during the first five years of life [19–21]. However, a previous study reported that 16% of patients presented an unexpected spontaneous resolution, suggesting that when SVAS is mild it is more likely to improve than to progress [22]. Peripheral pulmonic stenosis (PPS) occurs in approximately 30% of patients and tends to improve with age in the majority of patients [21, 22].

Systemic arteriopathy is present in approximately 20% of WBS patients and the affection of the elastic arteries can be a local or diffuse narrowing [23, 24]. The most frequent is the stenosis of the thoracic aorta (STA), which is a separate entity from coarctation of the aorta [25]. A study in a cohort of 270 patients with WBS found that STA was present in 14%, long-segment STA was more frequent than

discrete STA [25]. Many patients with STA presented other vascular stenoses; including neck vessels, renal artery, abdominal aorta, mesenteric and celiac artery [25]. It is important to diagnose systemic stenoses before a surgical intervention to avoid hypoperfusion of the organs irrigated by the stenotic arteries and because it can change the surgical technique used [23, 25]. Abdominal aortogram studies in WBS patients showed that the morphology of the abdominal aorta is characterized by segmental changes in the diameter, hypoplastic at the diaphragm level, suprarenal narrowing, renal artery stenosis (RAS) and with an increase in infrarenal aorta diameter [26]. Middle aortic syndrome (MAS); consisting of stenoses of the thoracic and abdominal aorta, mesenteric and renal arteries, has been described in 2-70% of cases [27, 28]. The higher incidence of other arterial stenoses in patients with STA might indicate that STA is a marker for worsened generalized arteriopathy [29]. RAS is present in 7-58% of patients [29], when associated with hypertension its etiology be considered renovascular [26]. CT or RM angiogram are good imaging techniques to study the vascular complications in WBS patients, diagnose MAS and control it after its surgical intervention [28].

Other frequent cardiovascular alterations reported in WBS, that can occur either isolated or accompanied by SVAS or PPS, are mitral valve disease, aortic coarctation, pulmonary or aortic valve disease or ventricular septal defect (Table 1)[12, 18, 21, 22].

Two studies reported that male patients had significantly increased severity of cardiovascular disease, including SVAS, compared to female patients [30, 31]. The authors attributed the increased severity to sex hormones, which are known to be potent regulators of cardiovascular development and function [30].

Clinical Finding	Combined Prevalence (%)	Range of Prevalence
SVAS	69	28-100
Pulmonary arterial stenosis	34	0-83
Hypertension	50	5-70
Mitral valve disease	15	4-43
Aortic coartation	4	0-19
Aortic hypoplasia	2	0-14
Pulmonary valve disease	5	0-47
Aortic valve disease	3	0-11
Ventricular septal defect	8	8-21
Atrial septal defect	3.5	3.5-6

Table 1. Cardiovascular findings in WBS individuals. Adapted from [12, 18, 21, 22, 25, 27, 28, 31–33, 255].

Arterial hypertension (HTN) can occur in approximately 50% of WBS individuals, although, as seen on Table I, this percentage is highly variable depending on the criteria and the methods used for blood pressure measurement [12, 16, 31–33]. A study using ambulatory blood pressure measurements (ABPM) found an increased incidence of HTN in WBS compared to the control group (40% vs. 14%); the difference was more striking when comparing children (46% vs. 6%). The authors also reported that WBS increased by 10 mmHg the mean daytime and nighttime blood pressures [33]. Another study using ABPM found a similar frequency of HTN, with a higher frequency of systolic HTN compared to diastolic HTN, and a blunted nocturnal decrease in normotensive individuals [34]. This finding was corroborated in a study evaluating exercise tolerance and ABPM, in which 47% of WBS patients did not have an adequate nocturnal blood pressure decrease [35]. A blunted nocturnal decrease is associated with increased end-organ damage and cardiovascular events [36, 37]. Some

of the causative mechanisms include an increase in adrenergic activity and decrease in vagal activity during sleep, increased sympathetic activity due to sleep disturbances or activation of the renin-angiotensin-aldosterone axis with volume excess [38]. A recent study using ABPM studied the behavior of central and peripheral blood pressures and arterial stiffness parameters in children with WBS using ABPM and found a higher nighttime heart rate and an impaired physiological reduction in the day-night shift suggesting an abnormal sympathetic cardiovascular control and an increase in small arteries resistance [39]. Regarding the pharmacological treatment of HTN, at the moment there is no information about which should be the first line of treatment for HTN in WBS patients [12, 32].

The high variability of the cardiovascular phenotype in WBS patients has been attributed to the presence of modifying factors [12], for instance gender as described previously [30]. A possible genetic modifying factor is *NCF1* copy number [31]. A study found that HTN was significantly less prevalent in patients whose deletion included *NCF1* [31]. Patients with only one copy of *NCF1* were protected against HTN by a reduction of angiotensin-II mediated oxidative stress [31], as it will be explained in greater detail in the final part of the introduction.

Left ventricular function and textural properties of WBS patients has only been analyzed in one study. The authors performed an echocardiography in a cohort of 16 patients with WBS that had SVAS or HTN and found left ventricular hypertrophy (LVH) in 56% of patients and abnormal left ventricular geometry in 62% [40]. WBS patients with LVH presented diastolic dysfunction compared to patients with a normal left ventricular mass [40].

The major cause of death of WBS individuals is secondary to cardiovascular complications. Patients with WBS have a 25-100 fold increased risk of sudden death compared to the general population [41]. The majority of causes of sudden cardiac death in patients with SVAS that occur during sedation or anesthesia are secondary to acute

myocardial ischemia [42]. Pham et al reviewed cardiovascular data from 242 individuals from the Pediatric Cardiac Care Consortium with WBS and found a 6% mortality rate (15/242) after cardiac surgery or catheterization. Patients presenting both SVAS and PPS had the highest mortality rate [43].

1.4 Endocrinological Phenotype

Endocrinological alterations described in WBS individuals include transient hypercalcemia of infancy, subclinical hypothyroidism and glucose intolerance.

Transient hypercalcemia in infancy has a frequency of approximately 15%, although its frequency ranges from 5-50% depending on the criteria and methods used to diagnose it [44, 45]. The episodes are usually asymptomatic, although patients can also present nonspecific symptoms such as hypotonia, irritability, decreased appetite and constipation[16]. In some instances, it can be accompanied by hypercalciuria that often persists during the first year of life [24, 45]. Nephrocalcinosis can be found in less than 5-10% of WBS patients undergoing a renal ultrasonography [16, 45–47].

Subclinical hypothyroidism, defined as an elevation of thyroid stimulating hormone (TSH) with normal thyroid hormone levels, can occur in approximately 15-30% of WBS individuals [48–50]. Several studies described that the elevation of TSH was age dependent, subclinical hypothyroidism being more frequent in younger patients. Some authors hypothesize that it could be attributed to the immaturity of the hypothalamic-pituitary-thyroid axis that spontaneously resolves with increasing age [49]. These studies also found that approximately 70% of WBS patients presented hypoplasia of the thyroid gland, the majority of them accompanied by functional alterations suggesting that hypothyroidism may be a consequence of reduced thyroid volume [48–50]. Clinical hypothyroidism is a rare in WBS but clearly more frequent than in the general population [48].

Abnormal glucose metabolism was observed in 75% of adult and in 63.6% of young adults with WBS after a 2-hour glucose tolerance test [51, 52]. Glucose metabolism alterations ranged from glucose intolerance to diabetes mellitus (DM). The authors of this study recommended the glucose tolerance test as the gold standard to diagnose glucose alterations in WBS individuals, since they did not find significant differences with respect to controls in fasting glucose, median insulin levels or hemoglobin A1C [51]. A recent study in young adults with WBS evaluated β-cell function and insulin sensitivity and found an association with impaired insulin sensitivity but not with β-cell function, islet autoimmunity or other traditional risk factors for type 2 DM (age, BMI or family history) [52].

1.5 Gastrointestinal Manifestations

Gastrointestinal manifestations are frequent findings at all ages. During infancy and childhood, frequent manifestations include feeding difficulties, gastrointestinal reflux, constipation, colic and failure to thrive [24].

Gastrointestinal reflux also occurs in approximately 25% of adults and responds well to medical treatment [44].

A frequent finding in adults is chronic abdominal pain that can have numerous causes, such as constipation, diverticular disease or discrete arterial stenoses that can result in bowel schemia [44]. Constipation occurs in approximately 50% of adults with WBS [11, 44], and sometimes can lead to hemorrhoids or rectal prolapse. It should be aggressively treated with a well balanced diet with high fiber content and in some cases with medication[7]. Diverticular disease occurs in 25 to 40% [11, 53] of adults with WBS at a younger age than in the general population, presumably secondary to elastin haploinsufficiency [44]. Approximately 75% of patients required surgical intervention [11]. Anxiety can also cause abdominal pain; however, this should be a diagnosis of exclusion [11].

Celiac disease is present in 2.2-10% of patients with WBS [54–56], much higher than the mean prevalence estimate in the general population (0.9%) [57]. Antibody screening for celiac disease (anti-endomysium and anti-gliadin) should be performed in WBS with clinical suspicion of celiac disease.

1.6 Neurological Phenotype

Neurological signs are present in 40 to 70% of WBS individuals of all ages [58, 59] and include central hypotonia, hyperreflexia of lower extremities accompanied with peripheral hypertonia [60]. Cerebellar signs include intention tremor, dysmetria, dysdiadokinesis and ataxia [60]

The most common central nervous system structural abnormality reported in WBS individuals is Arnold Chiari malformation type I [16]. Its exact prevalence is unknown since neuroimaging techniques (MRI) are not routinely performed on WBS patients. However, a study found that it was present in approximately 10% of WBS patients [61]. Acute symptoms include headaches, syncope and suboccipital pain secondary to flow obstruction of cerebral spinal fluid (CSF) [62]. If untreated, chronic posterior fossa compression can lead to upper extremity weakness, muscle atrophy and paresthesias [62]. It is recommended that WBS individuals presenting neurological manifestations should be evaluated by a neurologist and with neuroimaging techniques [44].

Neuroanatomical findings in WBS include a reduction of 10-15% compared to controls in overall cerebral volume, with relative preservation of cerebellar size [63, 64]. Cerebral volume reduction is at the expense of white matter; since grey matter and CFS seem to be spared [63, 64].

1.7 Cognitive and Behavioral Phenotype

The cognitive phenotype in WBS individuals is characterized by mild intellectual disability, with mean IQ scores of 50-60 (range 40-100) that remain stable throughout their lifespan [60, 65]. They have

relative strengths in concrete language, concrete non-verbal reasoning and verbal short-term memory and extreme weaknesses in visual spatial construction [66, 67].

Children with WBS present a delay in language acquisition; they develop strengths in concrete receptive and expressive vocabulary but difficulties in vocabulary for spatial, dimensional and temporal concepts [67], as well with the pragmatics of language, for instance maintaining the topic of conversation [67]. The most severe deficit is in visual spatial construction, evaluated with the use of complex drawings (Rey-Osterrieth Complex Figure) or pattern construction tasks [60]. Figure 3 compares their relative verbal strengths and visual spatial weakness when a 15-year old individual with WBS is asked to describe and draw an elephant [68].

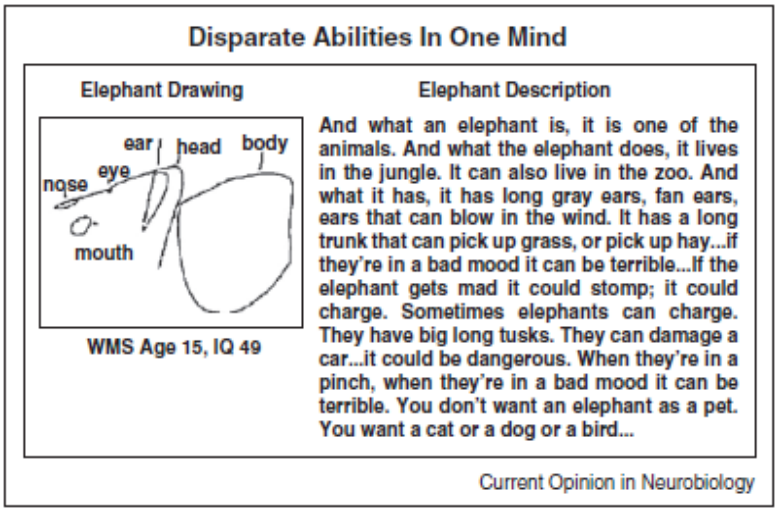

Figure 3. Relative strengths and weakness of WBS cognitive phenotype. Elephant drawing and description by a 15-year-old patient with WBS (IQ 49). From[68].

As it can be observed, the boy is able to explain and describe with great detail an elephant. However, when asked to draw it, he draws in detail all the parts of the elephant but is unable draw it as a whole.

Individuals with WBS have a unique social phenotype characterized by an overly friendly, gregarious and empathetic personality [60, 69, 70]. Infants and toddlers first manifestation of extreme sociability is the prolonged time they stare at human faces [60]. As can be observed in Figure 4, when given a task, instead of responding to the stimulus (object), they try to engage with the examiner [68, 71]. Patients with WBS have a propensity to focus at the faces, specifically at the eyes [72, 73].

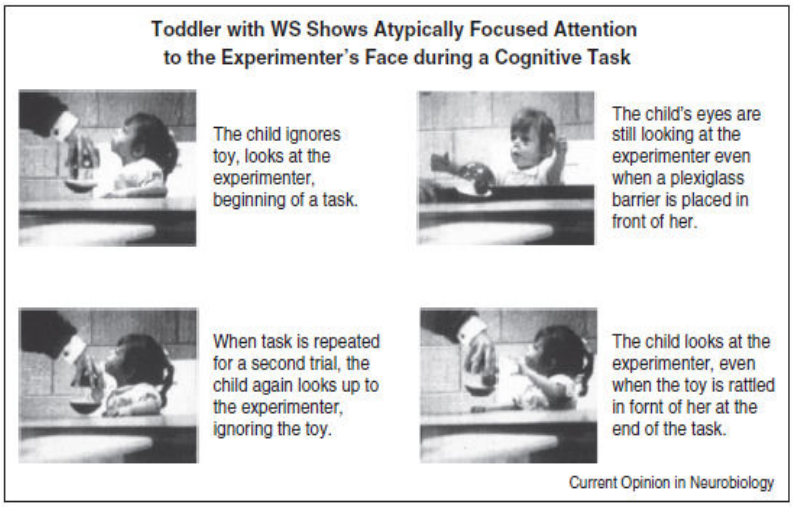

Figure 4. Atypically focused attention of a child with WBS to the examiner's face during a cognitive task. From [68].

Even though they have a tendency to engage in social interactions with strangers, a trait that is maintained throughout their lifespan [60, 74], many individuals have difficulties establishing and maintaining friendships [75]. Many adults are socially isolated, without significant relationships with the opposite gender or without stable social relations with peers [76].

Adaptive behavior refers to practical, conceptional and social skills that are learned for an individual to function independently in the community and it has been evaluated in WBS individuals by parent rated scales [60]. WBS individuals usually have higher performance scores in socialization and communication than in daily living and motor scales [77, 78]. A study conducted in adults with WBS found that approximately 16% were living independently, 38% had some form of job placement (part time jobs, low paid or voluntary), and few adults could handle their finance [79]. The majority of adults were able to take care of their basic self-hygiene such as dressing and using the lavatory. Approximately half of the adults were able to go alone to local shops or to familiar environments [79].

Psychiatric problems are also very frequent among WBS individuals; the main psychiatric diagnoses include attention deficit hyperactivity disorder (ADHD) and anxiety [80]. Approximately 30 to 70% of children with WBS meet DSM-IV criteria for ADHD [81, 82]. The majority of children were diagnosed with the predominantly inattentive type (69%), few with the combined type and only 4% with the predominantly hyperactive-impulsive type [81]. Approximately 60% of children with WBS meet DSM-IV criteria for at least one anxiety disorder; specifically phobia (54%), generalized anxiety disorder (12%) and separation anxiety (7%) [60]. In a longitudinal study, approximately 80% of adults with WBS presented anxiety disorder at some point during the study [83]. The examiners observed that anxiety was present at different ages and that symptoms persisted over long periods of time [83]. Episodes of anxiety did not resolve, but shifted towards new objects [83]. A recent study showed an association between higher intellectual abilities and higher anxiety levels [84]. Greater cognitive abilities allows them to be aware of potential threats which in turn can cause an increase in anticipatory anxiety [81].

1.8 Auditory Manifestations

WBS is associated with increased auditory problems. Approximately 90% of WBS individuals present hypersensitivity to sound (hyperacusia) or pain (odynacusis) that persists till adulthood [85]. It has also been described that approximately 50% of children with WBS present chronic otitis media [24]. Mild to moderate high frequency sensorineural or mixed hearing loss has been described in 60 to 70% of school-aged children with WBS possibly secondary to cochlear dysfunction [85–87]. Recurrent ear-wax build-up is common in adults with WBS [11].

1.9 Ophthalmologic Manifestations

Ophthalmologic manifestations are also frequent among individuals with WBS, including a stellate iris pattern in 70% (Figure 5) [88], high frequency of refractory errors (67% presented hyperopia, 20% astigmatism and 7% myopia) and strabismus in 37% of patients [89, 90].

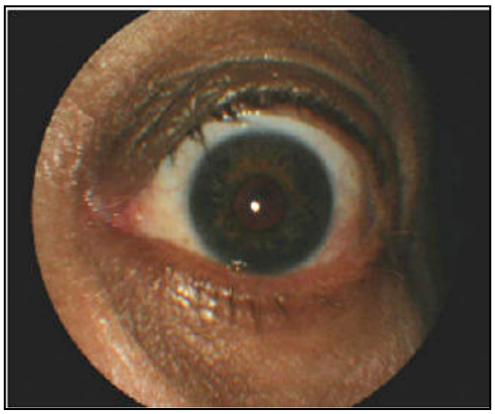

Figure 5. Stellate iris pattern of a patient with WBS (Modified from [90]).

1.10 Dental Manifestations

Individuals with WBS have a high frequency of dental problems; the most frequent are malocclusion (85%), microdontia (95%), hypoplastic enamel defects and abnormal tooth morphology [91]. In

adults with WBS a frequent problem is poor dental hygiene which can lead to caries and gum disease [11, 44].

1.11 Genitourinary Manifestations

Genitourinary structural abnormalities are present in 20 to 35% [11, 24, 53] of children with WBS and include renal ectopia, agenesis, hypoplasia, duplication, hydronephrosis and duplicated collecting system [46, 47, 92, 93]. Other frequent genitourinary manifestations are enuresis present in 50% of patients and recurrent urinary tract infections in 30% [24]. Bladder diverticulum has also been reported in WBS adults, and is also thought to be secondary to elastin haploinsufficiency [44].

1.12 Musculoskeletal Manifestations

A significant number of individuals with WBS have musculoskeletal manifestations. Approximately 90% of infants present significant hypotonia and joint laxity, which may contribute to the delay in ambulation [24, 44]. Around 60% of children adopt an awkward gait characterized by a posture of bent knees, flexed-hip stance to improve their stability [24, 44]. Radio-ulnar synostosis is present in approximately 20% of children with WBS [24]. With increasing age, individuals with WBS present joint contractures (50%), which tend to worsen overtime if they are not properly treated [44]. Kyphosis and lordosis are also a frequent finding, occurring in approximately 20 and 40%, respectively [24].

1.13 Integumentary system Manifestations

Individuals with WBS also present skin manifestations. Approximately 80% of adults older than 20 years have premature hair greying; the youngest individual in a recent study was 11 years old [94]. Soft skin was reported in 83% of individuals, with facial wrinkling in 92% of cases [94]. A relative common finding described in this study was dry skin accompanied in some cases with hyperkeratosis present in 20% of individuals. Onychodystrophy was observed in 22% of patients [94].

Approximately 40% of young children have had surgical repairs of inguinal hernias [44] and 15 -20% have umbilical hernias [44, 92].

1.14 Management and Genetic Counseling

Williams Beuren syndrome is a multisystemic disorder with variable expressivity. Patients should be followed-up in multidisciplinary consults to evaluate patients in a global manner, diagnose and treat symptomatology promptly and decrease the number of hospital visits.

At the moment of clinical suspicion a series of evaluations are recommended, including a complete physical and neurological exam, anthropometric data, cardiovascular evaluation (blood pressure and echocardiography), ophthalmological exam, calcium metabolism evaluation, a neuropsychological evaluation and a molecular test to confirm the presence of a 7q11.23 deletion [95]. When giving a diagnosis, it is recommended that it should be a clinical geneticist, who should inform about the sporadic genetic cause, the natural history of the disease, the recurrence risk and the importance of starting early intervention programs and school support [95].

Table 2 shows the recommendations for medical monitoring in WBS individuals [24, 44, 95]. As can be seen below, family support and contacting with patients associations is always recommended. Cardiovascular evaluation should include physical examination and echocardiography; the periodicity depends on the cardiovascular lesion. Basic audiologic evaluation should be performed at 30 years of age to rule out sensorineural hearing loss and since then it should be evaluated every five years [44]. Further evaluations depend on present symptomatology or previous clinical diagnoses or birth defects and other medical problems.

	Infancy (NB – 1 year)	Early Childhood (1 – 5 years)	Late Childhood (5 – 13 years)	Adolescence (13 – 18 years)	Adults
Family Support	X	X	X	X	X
Complete physical exam	X	X	X	X	Every 1 – 2 years
Growth feeding (growth charts)	X	X	X	X	X
Cardiology Evaluation	X	X	Every 1 – 2 years*	Every 1 – 2 years*	Every 1 – 2 years*
Blood Pressure	X	X	X	X	X
Ophthalmological Evaluation	X	X	Every year	X	Every year
Hearing Evaluation	X	X	Every year	X	Every 5 years*
Musculoskeletal Evaluation		X	X*	X	X*
Exclude Celiac Disease		X	X		
Renal Function		X	Every 4 years*	Every 4 years*	Every 4 years*
Calcium metabolism	X	X	Every 4 years*	Every 4 years*	Every 4 years*
Thyroid Function	X	X	X	X	Every 3 years
Glucose Metabolism				X	X
Diet high fiber content		X	X	X	X
Physical Activity			X	X	X
Development	X	X			
School Performance		X	X	X	X
Sexuality				X	X

Table 2. Recommendations for medical monitoring of individuals with WBS [24, 44, 95].

*Periodicity depends on patient's symptomatology or clinical diagnosis.

Genetic counseling to the families should include information regarding clinical manifestations and natural history of the disease, available management strategies, the molecular mechanism, recurrence risk for the progenitors, occurrence risk of first-degree family members and the proband´s risk of transmission. Williams-Beuren syndrome is caused by a recurrent deletion at chromosome 7q11.23 due to non-allelic homologous recombination (NAHR) during gamete formation. In the majority of cases it has a sporadic occurrence,

although few cases have been reported with an autosomic dominant pattern [96]. The recurrence risk for successive pregnancies for parents not carriers of susceptibility alleles is very low, approximately the same as for the general population, 1/7,500-1/10,000 [1, 95, 97]. If the progenitors are carriers of susceptibility alleles (copy number variants at 7q11.23 or paracentric inversion) the risk increases slightly, approximately 1/1000 [97]. First-degree relatives have the same occurrence risk as the general population.

WBS individuals have a risk of 50% of transmitting the deletion, and the disease, to their children [95, 97]. However, their intellectual and social abilities preclude most patients from having offspring, so that most cases present as sporadic occurrences.

2 WILLIAMS-BEUREN SYNDROME: CRITICAL REGION (WBSCR)

2.4 7q11.23 Genomic Architecture

The 7q11.23 WBS critical region has a complex genomic architecture (Figure 6). The single copy gene region is flanked by three large segmental duplications (SD) or low copy repeats (LCRs). SD or LCRs are large blocks, usually of 10-400 kb of genomic DNA, of highly identical sequence (>97%) generated during primate evolution that can predispose the region to genomic rearrangements [98]. The LCRs are located in the centromeric, medial and telomeric segment of the WBS locus; and each is composed of three blocks called A, B and C. The centromeric and medial duplicons are in the same orientation (tandem repeats) but different order. While the telomeric duplicon is in the opposite transcriptional orientation (inverted repeats) but in the same order as the centromeric block [97, 99, 100]. The three blocks share a very high degree of nucleotide homology (≈98-99%) [97, 99] that can lead to misparing and unequal crossover during meiosis causing deletions, duplications or inversions.

The single copy gene region is located between the medial B and C blocks and span an area of ≈1.2 Mb [97, 99, 100].

The A blocks contain four pseudogenes: *STAG3L*, *PMS2L*, *GATS-L* and a fragment with very high homology to *WBSCR19*. The medial block A, also contains the gene *WBSCR16*, which encodes an RCC-1 like G-exchange factor [99]. Its pseudogene is located at the telomeric block A [99].

The B blocks contain three genes: General Transcription Factor II-I (*GTF2I*, OMIM 601679), the GTF2I Repeat Domain-containing protein 2 (*GTF2IRD2*, OMIM 608899) and Neutrophil Cytosolic Factor 1 (*NCF1*, OMIM 608512). The functional copy of *GTF2I,* which encodes a transcription factor of the transcription factor family

TF-II, is located in the medial block Bm and its two pseudogenes (*GTF2IP1* and *GTF2IP2*), transcribed as a truncated protein, are located at the centromeric and telomeric blocks [99], respectively. The medial and telomeric copies of another member of the transcription factor family TF-II, *GTF2IRD2,* are fully transcribed, while the centromeric copy (*GTF2IRD2P*) is not expressed since it lacks the first two exons. *NCF1* encodes for the p47 phox subunit of the nicotinamide adenine dinucleotide phosphate-oxidase complex (NADPH) and the only active copy is located in the medial B block [101]. The centromeric (*NCF1B*) and telomeric (*NCF1C*) copies are pseudogenes with a 2-bp GT deletion at the beginning of exon 2 [31].

The C blocks contain four genes, the *POM121* transmembrane nucleoporin (OMIM 615737), the NOP2/SUN domain family member 5 (*NSUN5*, OMIM 615732), the Tripartite motif-containing protein 50 (*TRIM50*, OMIM 612548) and the FK506-binding protein 6 (*FKBP6*, OMIM 604839). *POM121* is expressed at both the centromeric and telomeric blocks, it is an integral membrane component of the nuclear pore complex and mediates the transport of macromolecules across the nuclear envelope [99]. The *NSUN5* ancestral gene is located at the medial block and may have a methyltransferase activity [99]. The other two copies, *NSUN5C* and *NSUN5B*, are located at the centromeric and telomeric copies and are transcribed as truncated proteins [100]. The *TRIM50* gene is located at each block and is expressed [99]. The ancestral copy of the *FKBP6* is located at the medial block, is involved in the synaptonemal complex involved in pairing and recombination of homologous chromosomes during meiosis [99], and is also expressed.

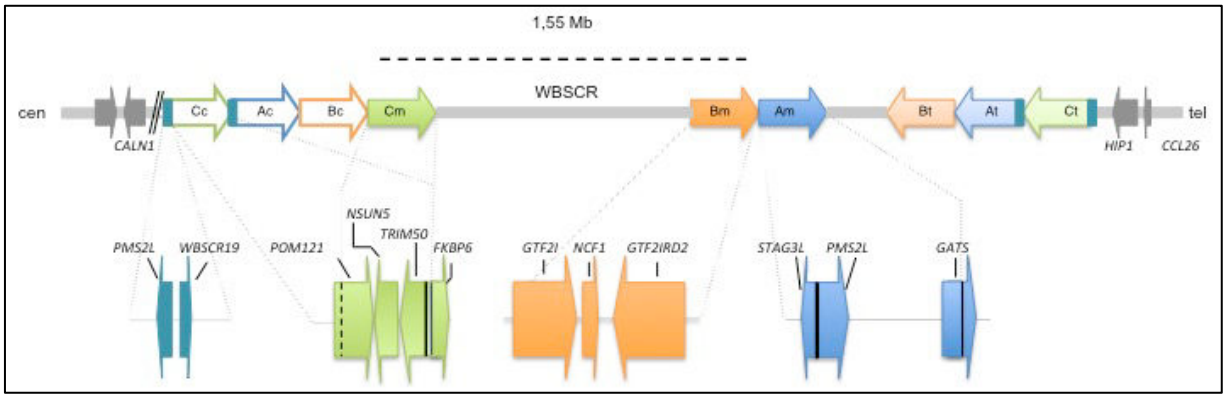

Figure 6. Schematic representation of the WBS Critical Region (WBSCR). Beneath the representation and gene content of each of the low copy repeats (LCRs) (in green the C blocks, in orange the B blocks and in blue the A blocks) (Modified from [276]).

2.5 Mutational Mechanisms

2.5.1 Non-Allelic Homologous Recombination

The characteristics of the genomic architecture can predispose to the occurrence of rearrangements and genomic instability [102, 103]. Non-allelic homologous recombination (NAHR) can result in recurrent rearrangements that include deletions, duplications or inversions [104]. Recurrent rearrangements are those that include the same genomic interval in unrelated individuals and the breakpoints usually lie in the LCRs flanking the event [105]. As shown in Figure 7, NAHR that occurs between duplicated sequences in direct orientation can result in deletion or duplication. While NAHR that occur between sequences aligned in an inverted position can result in inversions [106].

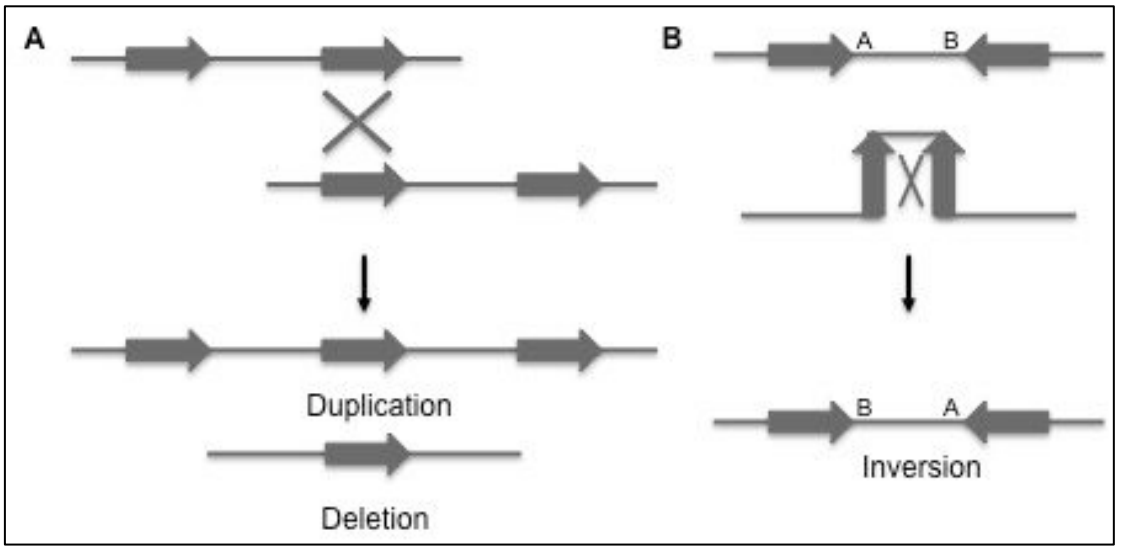

Figure 7. Schematic representation of non-allelic homologous recombination, between: **A.** Blocks in the same orientation can generate duplication or deletion. **B.** Blocks in an inverted position can result in an inversion (Modified from [107]).

NAHR can occur in three ways, intra-chromatidal crossover, intra-chromosomal or inter-chromatid crossover and interchromosomal crossover (Figure 8). When the mechanism is intra-chromatidal, only a deletion can be produced [106]. As shown below (Figure 8B), when an intrachromatid crossing over occurs it results in a deletion and a circular DNA molecule without centromere that that will not segregate during cell division [106]. Inter-chromatid or inter-chromosomal rearrangements will result in a reciprocal deletion or duplication [106] (Figure 8C).

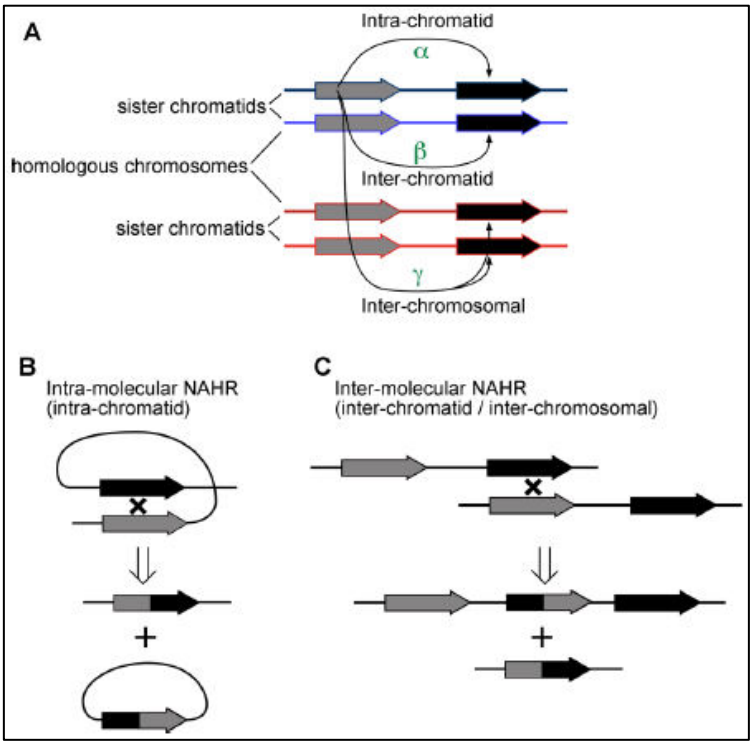

Figure 8. Mechanisms for non-allelic homologous recombination (NAHR). A. The three classes of NAHR are intra-chromatid, inter-chromatid and interchromosomal. **B.** Intra-chromatid NAHR can generate a deletion and a circular DNA molecule without a centromere. **C.** Inter-chromatic or interchromosomal NAHR can result in a deletion or duplication. From [106].

Various studies have been done to determine which factors can predispose to the rate of genomic rearrangements [106, 108, 109]. The rate of formation of genomic rearrangements is directly proportional to the degree and length of sequence homology, and indirectly proportional do the distance between them [108]. *Cis*-acting factors that can influence the recombination rate are the enrichment and formation of secondary structures such as G-quadruplexes, cruciforms, recombination motifs, *Alu* signal recognition, microsatellites and large A-T repeats [110–114].

In 2008, a 13-mer degenerative motif (5'-CCNCCNTNNCCNC-3') was discovered to be crucial for recruiting crossovers in 40% of all human hotspots [115]. This sequence is likely the binding site of the PR domain-containing 9 gene (*PRDM9*, OMIM 609760), that is a meiosis-specific histone H3 methyltransferase with a C-terminal tandem-repeat C2H2 zing finger domain encoded by a minisatellite [116]. *PRDM9* is likely to act as a *trans*-modifying factor for some recurrent genomic disorders, such as Charcot-Marie Tooth Type 1A (CMT1A) and hereditary neuropathy with liability to pressure palsies (HNPP)[117].

2.5.2 Paracentric Inversion

The paracentric 7q11.23 inversion is generated by meiotic or mitotic intra-chromatid misalignment between inverted homologous centromeric and telomeric LCRs followed by NAHR [97]. As shown in Figure 9, the crossing over can occur at each of the LCR blocks resulting in an inversion of variable size (1.8-2.6 Mb) [97]. The breakpoints of the inversion are external to the WBS single copy gene region, not disrupting any actively expressed genes, therefore inversion carriers do not present an abnormal phenotype [97]. The frequency of the heterozygous inversion in the general population is 5% [118], while approximately 30% of progenitors of patients with WBS are heterozygous for this inversion [97, 118, 119].

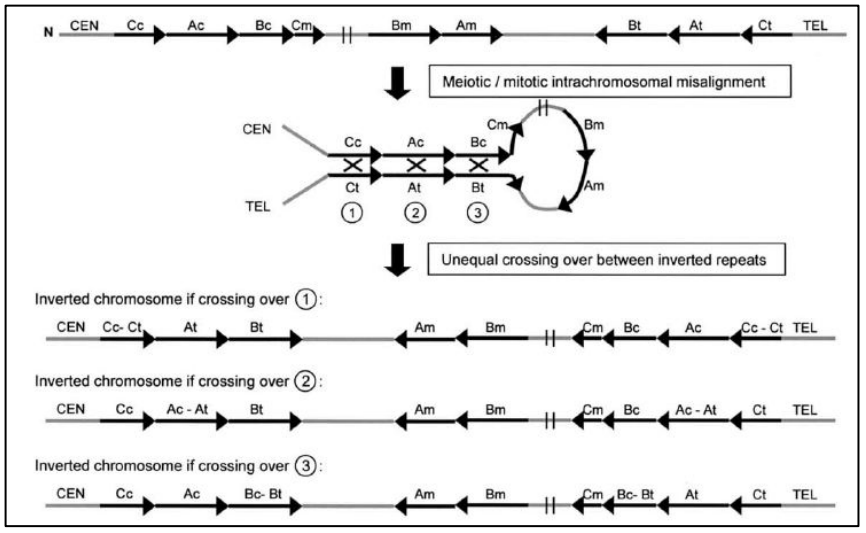

Figure 9. Schematic representation of the origin of 7q11.23 paracentric inversion. During meiosis or mitosis an intra-chromosomal misalignment occurs between inverted low copy repeat blocks (LCRs). Non-allelic homologous recombination (marked in X) can occur between any of the centromeric and telomeric blocks of LCR, originating an inversion of variable size. From [97].

The presence of the inversion can predispose to chromosomal misalignment during meiosis, resulting in reciprocal duplication or deletion [97].

2.5.3 Deletion

WBS deletion is mediated by NAHR between misaligned blocks of segmental duplications. As shown in Figure 10, approximately 90% of WBS deletions occur due to a crossing over between the centromeric and medial blocks B and has a size of 1.55 Mb. In the remaining 10% of cases the deletions occurs due to a crossing over event between the centromeric and medial blocks A and has a size of 1.8 Mb [97].

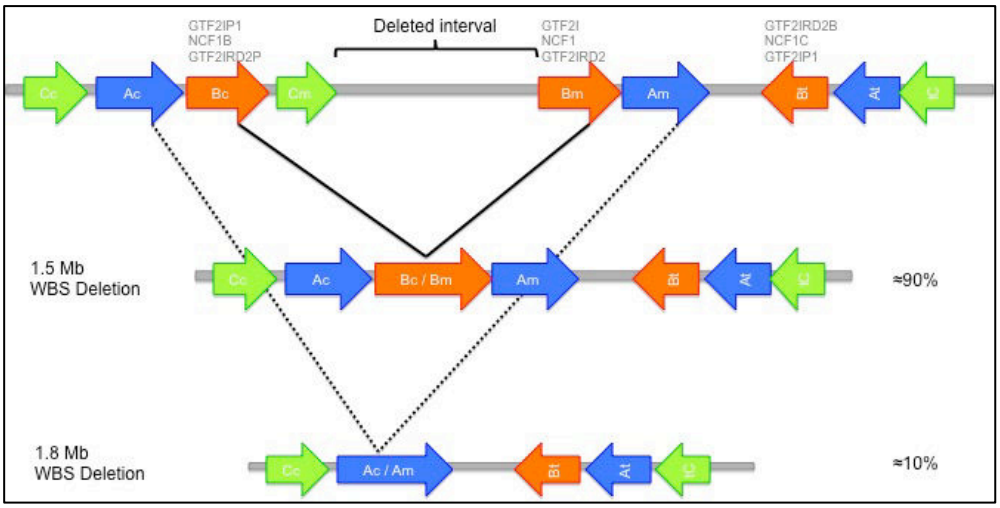

Figure 10. Schematic representation of the WBS deletion. (Modified from [97]).

The deletion occurs more frequently between the blocks B because of their higher degree of homology compared to that of the blocks A (99.6% compared to 98.2%) and because of the shorter genomic interval between the centromeric and medial B blocks (1.55 Mb) compared to the centromeric and medial A blocks (1.84 Mb) [97].

Approximately 25% of the 1.55 Mb deletions occur secondary to inversion-mediated events. As shown in Figure 11, the inverted and the normal chromosome 7 form a structural microloop in the middle of the inverted segment to achieve a complete sequence pairing [97]. The crossing over events leading to an inversion-mediated deletion are all located within the last 38 kb of block Bt and Bm, that are absent in block Bc [97].The crossing over can occur between the block Bt from the inverted chromosome and Bm of the normal chromosome (type I) or between block Bm of the inverted chromosome and Bt of the normal chromosome (type II) [97]. Recombination at any other site will lead to an acentric or dicentric chromosomes 7 that will not be able to segregate correctly during meiosis [97].

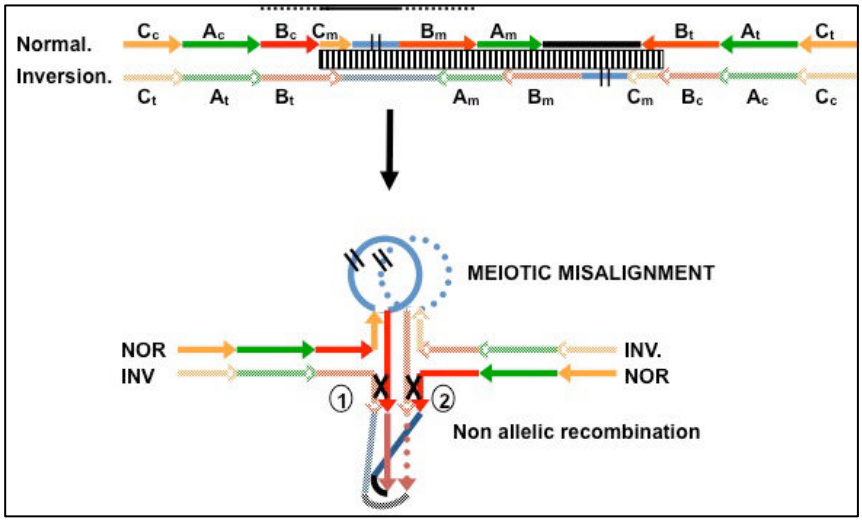

Figure 11. Schematic representation of the meiotic misalignment and crossing over event between an inverted and a normal chromosome. Type 1 unequal crossing over occurs between the block Bt from the inverted chromosome and Bm of the normal chromosome. Type II unequal crossing over occurs between block Bm of the inverted chromosome and Bt of the normal chromosome (Modified from [97]).

Approximately two-thirds of the deletions arise by interchromosomal rearrangements, while a third of cases are intra-chromosomal [97, 120]. However, all inversion mediated deletions occur after an inter-chromosomal crossover [97].

The molecular characterization analysis includes the study of single and multiple-copy microsatellites to determine the size and parental origin of the deletion [97]. To further define the breakpoint of the deletions, site-specific nucleotides (SSNs) are genotyped [97]. SSNs are nucleotides that are different between the LCRs blocks. By PCR amplification followed by digestion with restriction enzyme and size fractioning in agarose gels, the relative intensities of the products are quantified and a dosage quotient is calculated to determine a gain or loss of specific blocks [97]. Bayes et al [97] analyzed 34 putative B block SSNs and selected 13 on the basis of their high degree of specificity (Figure 12B).

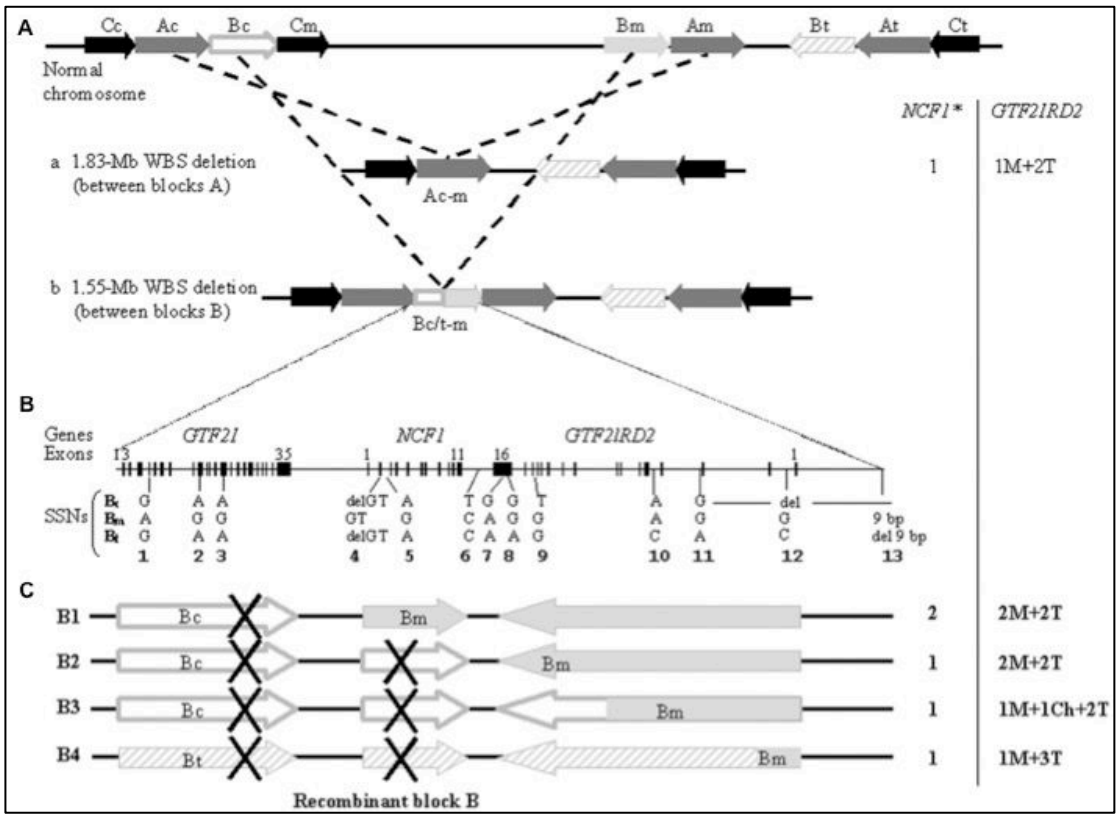

Figure 12. Schematic representation of the 1.55 Mb and 1.83 Mb deletions and breakpoint characterization. A. Representation of the 7q11.23 region, in black are represented the blocks C, in grey the A blocks and in white the B blocks. The top of the figure depicts the 1.83 Mb and 1.55 Mb deletions. **B.** Representation of the genomic content of the B blocks, as well as the location of the 13 genotyped site-specific nucleotides (SSNs) to refine the breakpoints of the deletion [97]. **C.** Scheme of the different locations of the 1.55 Mb deletion. In breakpoint 1 (B1) the crossover occurs at *GTF2I*, therefore *NCF1* gene content and the functional copy of *GTF2IRD2* are not affected. In B2, the breakpoint is located at *NCF1*, therefore the patient presents with only one functional copy but *GTF2IRD2* remains the same. In B3, the breakpoint occurs at *GTF2IRD2* generating a chimeric copy with the final exons belonging to the centromeric block and the initial exons belonging to the medial block. Finally B4, which is the product of the inversion-mediated deletion, has a loss of *NCF1* gene and a gain of the telomeric *GTF2IRD2* copy. From [31].

As shown in Figure 12, the 1.55 Mb deletion can occur at different points along the block B. When the crossover occurs at the *GTF2I*

gene, the two functional copies of *NCF1* and the two medial and telomeric copies of *GTF2IRD2* (2T+2M) are kept (Breakpoint 1, B1). When the crossover takes place at *NCF1* gene, there is a loss of a functional copy of the gene but *GTF2IRD2* telomeric and medial copies are maintained (2T+2M). In the third case, the breakpoint occurs at *GTF2IRD2* and there is a loss of NCF1 gene and the creation of a chimeric copy of *GTF2IRD2*, with the final exons belonging to the centromeric copy and the initial exons belonging to the medial copy. Finally, the fourth scenario is the inversion-mediated deletion, were there is a loss of *NCF1* gene and a gain of the telomeric copy of *GTF2IRD2* [31, 97]. In the 1.83 Mb deletion, the crossover occur between the centromeric and medial A blocks, result in a loss of *NCF1* gene and the medial copy of *GTF2IRD2* (1M+2T) [97].

Secondary to gene conversion events between *NCF1* gene and its pseudogenes, approximately 15% and 1% of individuals with WBS present three or four copies of *NCF1,* respectively [97, 121].

2.5.4 Duplication

The NAHR mechanism predicted the existence of the reciprocal 7q11.23 duplication. However, it was not until 2005 that the first case was described [122]. Possible explanations for the relatively recent description are that the phenotype of the reciprocal duplication is very different from the deletion, it is difficult to predict the possible phenotype associated to the duplication based on the knowledge of the gene content of WBS and, as has been observed, there is a great phenotypical variability among individuals [123, 124]. Moreover, detecting adjacent duplications by FISH can be challenging.

The 7q11.23 duplication (OMIM 609757) has an estimated population frequency of 1/13,000 – 1/20,000 individuals [124]. It is less frequent than WBS, since the duplication can only occur due to inter or intra-chromosomal mechanisms [106]. The clinical description includes dysmorphic features (prominent forehead, broad nose, deep-set eyes, straight eyebrows, thin lips, short philtrum), developmental delay with variable speech delay, behavioral problems, autistic features, hypotonia,

cardiovascular manifestations (aortopathy) and other congenital abnormalities [122–127]. In several reported cases, the duplication was transmitted from one of the progenitors and the majority of progenitors had previous history of speech delay and/or learning difficulties [124, 126]. However, there were also few progenitors who presented with normal speech and cognitive abilities [126]. There are two possible explanations for the lack of phenotype in parents, either because the neurocognitive problems might improve over time or because the studied progenitors presented a milder phenotype [126]. The duplication has been found in 0.1 to 0.12% of patients referred for cytogenetic testing for developmental delay, congenital malformations and autism [124, 128, 129] and in 0.076% of patients diagnosed with schizophrenia [130].

2.5.5 Triplication

The first case of a 7q11.23 triplication was recently described in 2010 [131]. The patient presented intellectual disability, severe expressive language delay, behavioral problems and dysmorphisms [131]. The triplication did not encompass the entire WBS critical region; since *FZD9* and *FKBP6* were not included [131]. Since the breakpoint was not located at the segmental duplications, it is possible that another mechanism different from NAHR could have mediated the triplication [131]. The phenotype of the triplication was more severe than the phenotype spectrum of the duplication, indicating a possible gene-dosage effect [131].

2.5.6 Copy-Number Variants

Due to the complex genomic architecture, the 7q11.23 region is prone to different types of rearrangements. Structural copy number variants, including deletion and duplication of the LCRs flanking the WBSCR, were identified in a cohort of WBS progenitors [132]. Investigators found deletions of LCRs in 4.44% and duplications in 2.22% WBS-transmitting progenitors [132]. When studying the presence of both polymorphisms in control individuals, they found that they were present in approximately 1% [132]. This indicates that not only the

7q11.23 paracentric inversion acts as a predisposing allele for WBS, but also the type of CNVs present in the region [132].

2.5.7 Novel technologies used to refine breakpoints

Conventional technologies used to refine breakpoints of recurrent genomic disorders required the use of several techniques. Pulsed-field electrophoresis followed by genomic Southern blot hybridization was used to obtain an atypical hybridization band with the breakpoint of interest [133]. This method required the preparation of high molecular-weight DNA, the identification of informative restriction enzymes and the design of probes that flank the deleted interval [134]. Although reliable, this approach was laborious and time-consuming.

Novel technologies available for breakpoint refinement include array comparative genomic hybridization (array CGH) and targeted capture of informative regions using multiple inversion probes (MIPs) followed by next generation sequencing (NGS) [134].

aCGH is based on the co-hybridization of sample DNA and control DNA marked with different fluorochromes to a solid support that contains DNA probes [135]. The hybridization competition allows detecting gains or looses of genetic material of the patient sample in comparison with the control sample [135]. aCGH became the standard for CNV detection, usually applied for an initial refinement of the breakpoint location [136, 137]. After an aCGH, it is required to perform a long-range PCR, subcloning and capilar sequencing to determine the precise location of the breakpoint [134]. Another approach is to use an ultra-high density custom oligonucleotide array targeted for the specific region of interest which refines the breakpoint location [138, 139]. This strategy is very useful for breakpoint that map in single-copy regions. However, is not very accurate for breakpoints that map within SDs of very high homology. The difficulties are due to probe cross-hybridization; in some cases the best refinement obtained is a region of approximately 100 kb of nearly identical sequence [134]. Long-range PCR is also relatively ineffective over long stretches of very high sequence identity [140].

Next generation sequencing (NGS), also known as massive parallel sequencing (MPS), is another approach. This technique allows the parallel sequencing of million of DNA fragments. Although more costly compared to other techniques, it has a less turn-around time with a high resolution. However, there also disadvantages when used to define breakpoints located within SDs, including short library inserts, short read lengths that do not reach both extremes of the breakpoint and the presence of copy number polymorphisms that can confound the location of the breakpoint [134]. This approach can be used using multiple inversion probes (MIPS) to capture the region of interest [134]. MIPs are short circularizable oligonucleotides (70-80 bp) used to capture specific genomic targets of less than 200 bp in legth [134]. The end of the probes are complementary to two different regions in the genome separated by one or more nucleotides, which is the variant or sequence that is going to be genotyped [141]. It has a high degree of specificity because it uses enzymatic steps and many loci can be studied simultaneous (approximately 3000 MIPs can be combined in a single simultaneous reaction) [134, 141]. The MIPs are targeted to capture paralogous sequence variants (PSV) [134]. Its accuracy depends on the density and spatial distribution of PSVs [134]. However, this technique also has some difficulties. Not all PSVs can be sequenced by MIPs, high or low GC content can affect the capture and high-copy repeat regions cannot be specifically captured [134].

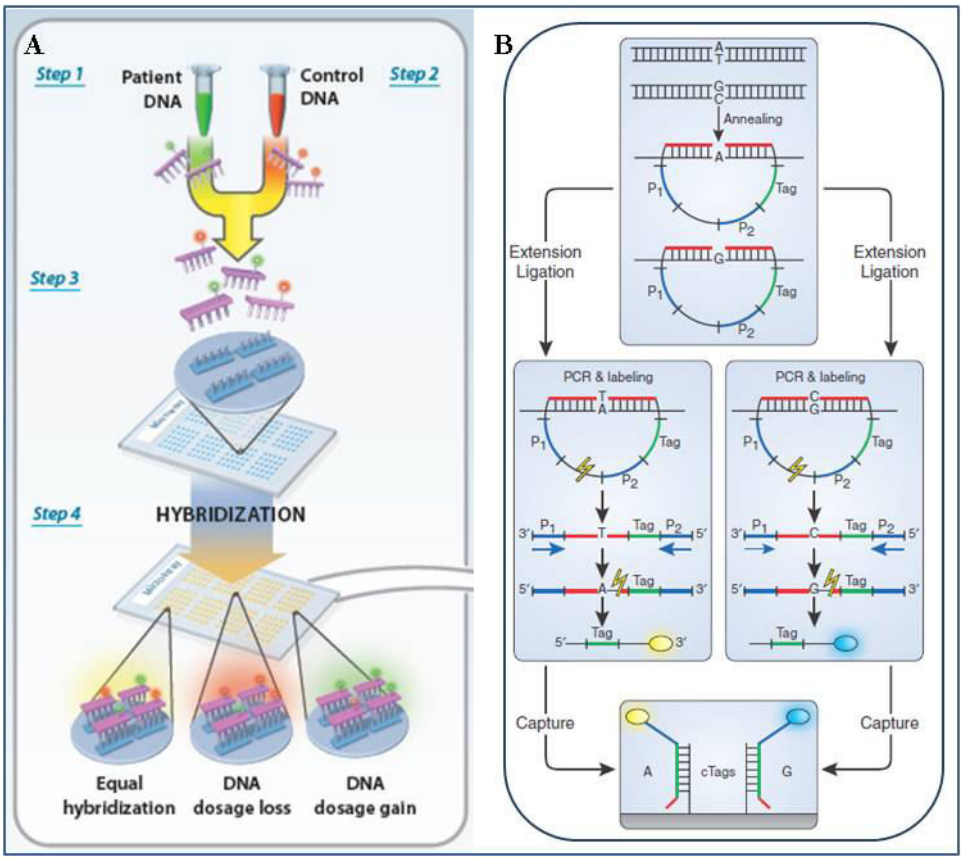

Figure 13. Schematic representation of novel technologies used to refine breakpoint location. A). Array comparative genomic hybridization (aCGH)**. B)** Next generation sequencing (NGS) using multiple inversion probes (MIPs) to capture the region of interest. (Modified from [142, 143]).

3 GENOTYPE – PHENOTYPE CORRELATION

It is difficult to establish a clear genotype-phenotype correlation in genomic disorders, since more than one gene can contribute to a given phenotype. Different approaches have been used to gain insight into the phenotypical consequences of the deleted genes. The study of atypical deletions at 7q11.23 can help refine the genes involved in a certain phenotype [144–150]. However, their analysis is complicated since these types of deletions are very rare and few deletions exist with similar breakpoints and sizes. In some cases, the exact breakpoint has not even been determined. With regards to their phenotype, different professionals have evaluated the majority of patients; using different clinical, psychological or cognitive tests; making their comparison even more complicated. Figure 13 shows the typical and atypical 7q11.23 deletion that have been reported previously, as well as the presence or absence of some of the main clinical features [150].

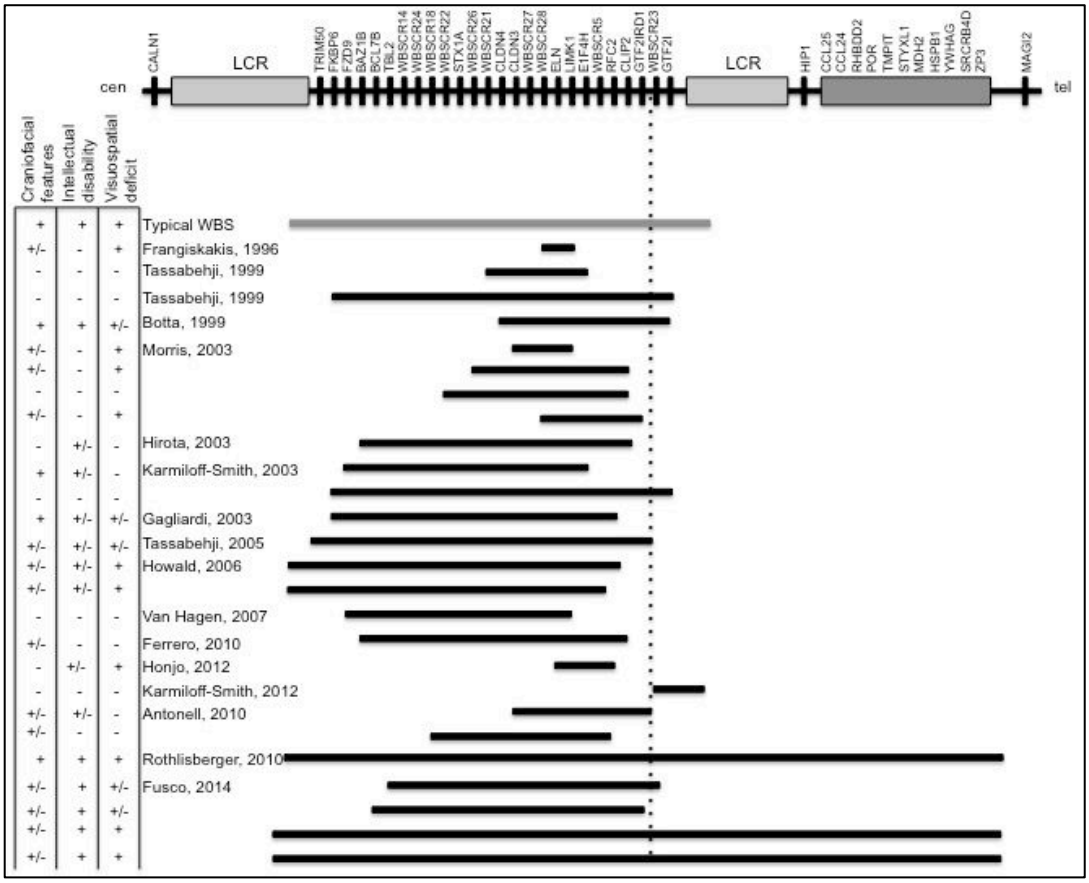

Figure 14. **Schematic representation of typical and atypical 7q11.23 deletions.** On the left, the main clinical characteristics, including craniofacial features, developmental delay, and visual spatial construction deficits (Modified from [150]).

The generation of murine mouse models is an alternative method to study concrete phenotypes, it allows tissue specific studies at different moments of development and test novel therapeutic approaches to improve or even rescue given phenotypes. The WBS region in mice shows a considerable degree of conserved synteny with the human region [151, 152]. The WBS region is located on chromosome band 5G2 in inverse orientation with respect to the centromere (Figure 14) [151, 152]. Several single gene knock-out models have been generated for a number of genes, including *Gtf2ird1* [153–155], *Eln* [156–158]or

Mlxipl [159]. Partial and complete deletions models that recapitulate most of the features of WBS have also been created [160, 161].

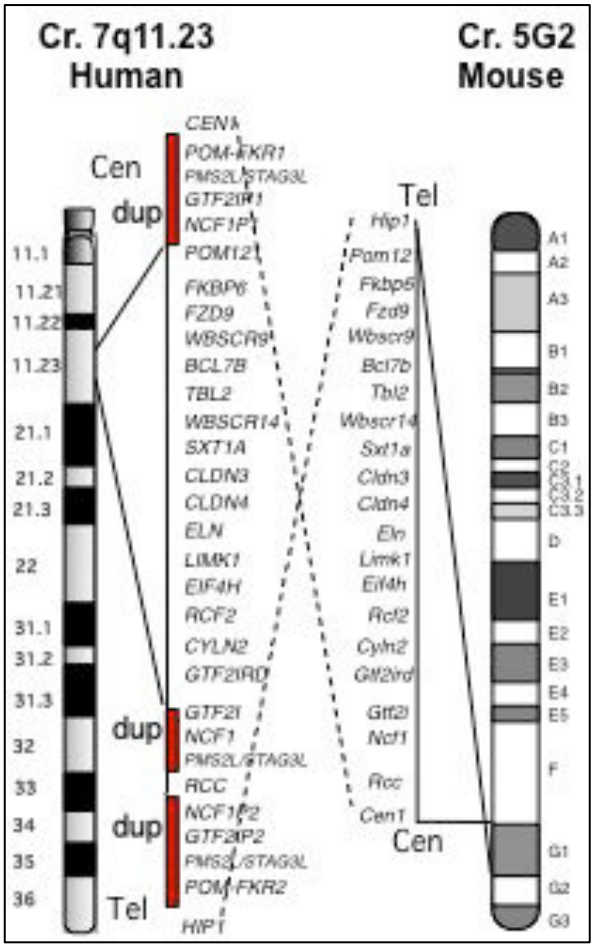

Figure 15. Schematic representation of WBS critical region at chromosomal band 7q11.23 in humans and in chromosomal band 5G2 in mice[151, 152]. There is a high degree of conserved sinteny, in mice the WBS region is in inverse orientation with respect to the centromere [151, 152] (Modified from [277]).

The proximal deletion model (PD) involving *Gtf2i* to *Limk1*, recapitulates the social phenotype associated to WBS, including

reduced social fear and gregariousness [162], suggesting that genes telomeric to the elastin locus might me involved in the WBS social phenotype. The distal deletion (DD) model includes *Limk1* to *Fkbp6* [160, 162] and although it includes a greater number of genes, it was associated with less obvious phenotypes [162]. Mice presented craniofacial abnormalities, including decreased cranial volumes, and also with connective tissue abnormalities such as hernias [162]. This is not unexpected since this deletion includes the elastin locus. With regards to the cardiovascular manifestations, DD mice presented 10-20% increase in mean blood pressure and histological sections showed disorganized and fragmented lamellar units in the blood vessel walls [160]. A double heterozygous mice (D/P) was also studied and it presented similar manifestations as the partial deletion models, however this model presents a homozygous deletion of *Limk1* [160]. A complete deletion (CD) mice was created encompassing the entire WBS critical region [161]. CD mice present decreased body weight at birth, decreased brain weight and a milder cardiovascular phenotype compared to DD, including mild increase in mean blood pressure, mild increase in arterial wall thickness and cardiac hypertrophy [161].

Figure 15 shows what is known about genotype – phenotype correlations in WBS based on gene function, atypical deletions and single and multiple-gene murine models. In the following paragraphs it will be discussed in greater detail.

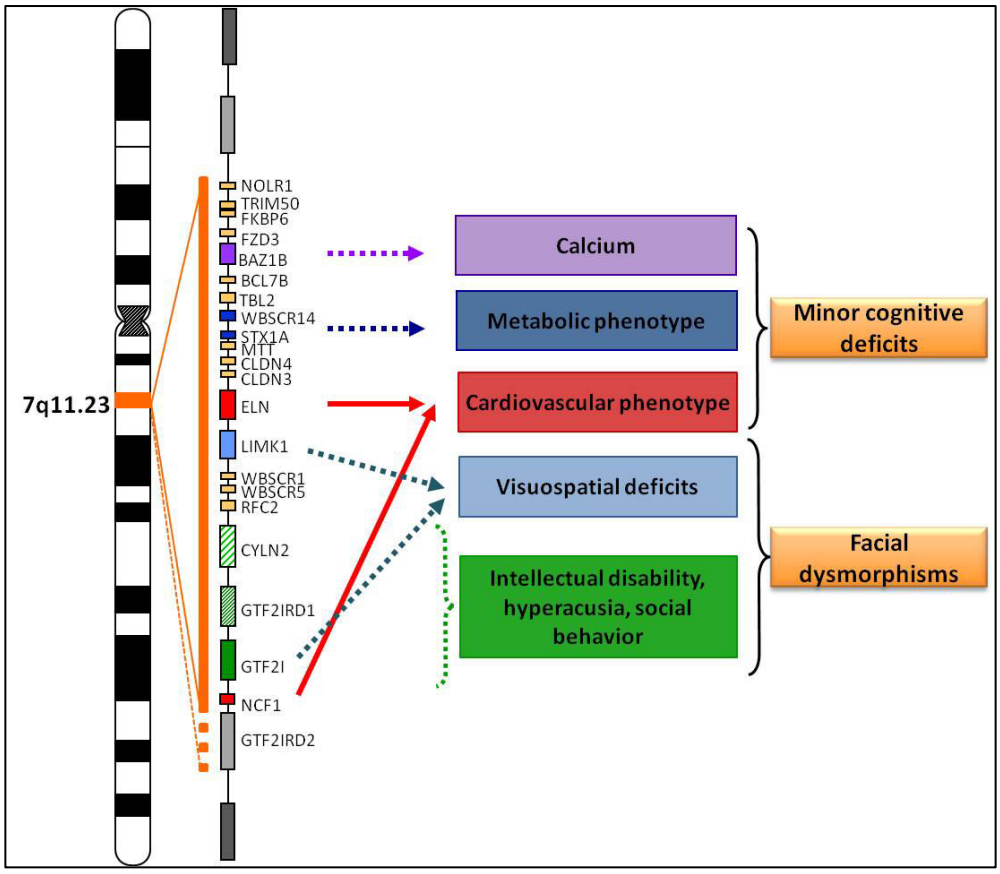

Figure 16. Schematic representation of WBS critical region at chromosomal band 7q11.23. Genotype-phenotype correlations are marked in colors. Evidence from atypical deletions are marked in orange, centromeric genes have been associated with minor cognitive deficits while telomeric genes have been associated with facial dysmorphisms.

3.4 Cardiovascular phenotype

The cardiovascular phenotype has been associated with the *ELN* gene and the *NCF1* gene.

3.4.1 Elastin

Elastin (*ELN*, OMIM 130160) was the first gene to be linked with a specific phenotype; it is responsible for the cardiovascular and connective tissue abnormalities observed in WBS patients. In 1993, it was found that a t(6;7)(p21.1;q11.23) translocation disrupted the elastin locus and co-segregated with non-syndromic supravalvular aortic stenosis suggesting it as the cause [163]. Since then it has been shown that point mutations, deletions or translocations that disrupt the elastin locus can cause non-syndromic supravalvular aortic stenosis (OMIM 185500) [163–166].

Elastin is the main component of the arterial extracellular matrix [158]. It is mostly expressed during the third trimester of gestation and the first postnatal years. Elastin is synthesized by smooth muscle, it is secreted as a monomer, tropoelastin, and after post-translational modifications it is organized into insoluble polymers that form concentric rings of elastic lamellae around the arterial lumen (Figure 15) [167, 168].

Elastin is a potent autocrine regulator of vascular smooth muscle activity, it induces actin stress fiber organization, inhibits proliferation, regulates migration and signals via a integrin, heterometric G-protein coupled pathway [158].

The single gene mouse model was created in 1998 and also presented cardiovascular manifestations. Mice lacking elastin died soon after birth due to obstructive arterial disease secondary to smooth muscle proliferation in the arterial wall [156]. Heterozygous mice had increased blood pressure, decreased aortic compliance and mild heart hypetrophy. Histologic analysis of ascending and descending aorta of these mice showed thinner elastic lamellae but with an increased number of lamellar units [157, 158].

3.4.2 Neutrophilic cytosolic factor 1

The neutrophilic cytosolic factor 1 gene (*NCF1*, OMIM 608512), located at the medial B block, encodes the $p47^{phox}$ subunit of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex. Mutations in *NCF1* cause granulomatous disease (OMIM 233700)[169].

The NADPH oxidase complex is composed of five subunits, the $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, $p22^{phox}$ and $gp91^{phox}$[170, 171]. Vascular NADPH oxidase is regulated by humoral growth factors such as cytokines and vasoactive agents, and by physical factors, including stretch and shear stress [172]. The $p47^{phox}$ subunit has a major role in activating the NAPDH oxidase complex [173]. NADPH oxidase enzyme generate oxidative stress by the conversion of oxygen to O_2^- [171].

The role of oxidative stress in essential hypertension has been studied in great detail and there are indirect evidence supporting its association with increased blood pressure, including increased plasma and urine levels of oxidative stress markers, increased vascular production of superoxide anion, decreased plasma levels of antioxidant vitamins and anti-hypertensive drugs reduce reactive oxygen species production and decrease oxidative stress by inhibiting the action of NADPH oxidase [174].

In humans the modifying effects of *NCF1* copy number on cardiovascular phenotype have been demonstrated [31, 175]. Patients presenting only one functional copy had a lower risk of presenting arterial hypertension compared to patients with two or more copies [31]. Cell lines of patients with only one copy had decreased $p47^{phox}$ protein, decreased superoxide anion product and lower protein nitrotyrosination, indicating that the loss of a functional copy of *NCF1* protects against hypertension by reducing angiotensin II-mediated oxidative stress [31]. A recent study also showed that the loss of *NCF1* was associated with reduced vascular stiffness [175].

Later studies in mouse models confirmed previous findings. The $p47^{phox}$ knock-out mouse model did not present hypertension when induced with angiotensin II and had lower oxidative stress production, vascular hypertrophy and endothelial dysfunction when compared to wild type mice [176]. The cardiovascular phenotype for the distal deletion mice were partially rescued when decreasing *Ncf1* gene dosage [177]. Double heterozygous mice for the distal deletion and a loss of function of *Ncf1* presented normal blood pressure, reduced angiotensin II levels and decreased heart size [177].

3.5 Neurocognitive and craniofacial phenotype

The neurocognitive and craniofacial phenotype in WBS has been associated to various genes, including members of the transcription factor II-I family (*GTF2I*, *GTF2IRD1* and *GTF2IRD2*), *LIMK1*, *CLIP2*, *BAZ1B*, *FZD9*, *STX1A* and *EIFH4H*.

3.5.1 Transcription Factor II-I Family

General transcription factor II-I (*GTF2I*, OMIM 601679), GTF2I repeat domain-containing protein I (*GTF2IRD1*, OMIM 604318) and GTF2I repeat domain-containing protein II (*GTF2IRD2*, OMIM 608899) are members of the transcription factor II-I (TFII-I) family of proteins characterized by the presence of multiple helix-loop-helix – like domains known as I repeats [178]. TFII-I family has been implicated in several important biological functions including cell cycle and proliferation, growth factor (TGF-β), calcium and immune signaling [179].

GTF2I is widely expressed early in development and in a uniform manner in the brain [180]. It is present exclusively in neurons, with the highest levels of expression in cerebellar Purkinje cells and hippocampal neurons [180]. *GTF2IRD1* is ubiquitously expressed in adult and fetal tissue, except for leucocytes [181], with an increased expression in the granular cell layer of the olfactory bulb, cerebellar Purkinje cells and neurons in the piriform cortex [180, 182].

GTF2I and *GTF2IRD1* have been proposed as potential candidates genes involved in the craniofacial, social and neurocognitive phenotype observed in WBS individuals. Individuals with smaller atypical deletions not including *GTF2I* or *GTF2IRD1* do not present the facial gestalt of WBS individuals, nor the social personality and visual spatial deficits [145, 147, 183]. An individual carrying a smaller deletion including all three TFII-I family members and 14 genes in the distal region exhibited autistic traits and the Williams Syndrome Cognitive Profile (WSCP) including visual spatial deficits corroborating their involvement [146]. Deletions involving only *GTF2IRD1* have increased our knowledge about its individual contribution [144, 184]. There are two cases reported in the literature, both present craniofacial dysmorphisms and borderline to normal cognitive abilities. However, they are discrepant in the overly-friendliness and specific visual spatial impairments [144, 184].

There are several *Gtf2i* murine models. Homozygous mutant mice generated by gene targeting with an inframe deletion of exon 2 presented decreased viability and fertility, and abnormal craniofacial morphology [185]. Heterozygous mice presented milder craniofacial alterations and some neurobehavioral alterations (decreased exploratory activity and anxiety) and a low threshold for sound intolerance [185]. A second model created by mutagenesis with a gene trap insertional vector presented similar characteristics. Homozygous mice died during embryiogenesis and presented neural tube defects. Heterozygous mice did not present relevant dysmorphic traits, but presented altered social behavior and increased social interaction [186]. Finally expression analysis of murine lines with *Gtf2i* and *Gtf2ird1* inactivation showed decreased expression of craniofacial related genes [187].

Several *Gtf2ird1* murine models have been created with different genetic backgrounds, which could explain their phenotypic differences. At one end of the spectrum, mice present facial dysmorphism similar to WBS individuals (periorbital fullness and a short snout) [154, 188] and at the other end, mice present a more severe phenotype, including

embryonic lethality, neural tube and vascular defects [155]. With regards to their behavioral phenotype, mice presented decreased fear and aggression, and increased social behaviors [189], a hypoactive-anxious phenotype, with gait and sensory motor abnormalities [153, 190].

GTF2IRD2 is not always deleted in WBS, it depends on the breakpoint and size of the deletion. Although its function is still unknown, it was observed that *GTF2IRD2* interacts directly with *GTF2I* and *GTF2IRD1* and that it regulates its activities by direct protein interactions and sequestration of the proteins to a novel nuclear compartment [191]. *GTF2IRD2* is expressed in human fetal and adult brain, and with *in silico* expression analysis it was determined that it is specially expressed in the cerebellum, orbitofrontal cortex and dorsolateral prefrontal cortex [192].

A study comparing cognitive, behavioral and psychological functioning between individuals with a 1.55 Mb deletion and with a 1.83 Mb deletion (including *GTF2IRD2*) found that individuals with the larger deletion presented increased impairments in spatial functioning, social reasoning and cognitive flexibility[192]. The authors attributed these differences to the haploinsufficiency of *GTF2IRD2* [192]. However, further studies are needed to corroborate this finding.

3.5.2 LIM Domain Kinase 1

LIM Domain Kinase I gene (*LIMK1*, OMIM 601329) is involved in the reorganization of the actin cytoskeleton by phosphorylating and inactivating the protein cofilin [193]. Reorganization of the actin cytoskeleton plays an important role in regulating axon formation [194]. *LIMK1* is expressed during embryogenesis in the central nervous system, including the inner nuclear layer of the retina, the developing spinal cord, the cranial nerve and the dorsal root ganglia[193]. *LIMK1* has been shown to promote axon out-growth and deliver proteins to growth cones involved in the development of neural polarity [195].

LIMK1 has been proposed as a contributing factor to impaired visual spatial constructive cognition. However, evidence from smaller atypical deletions has been inconclusive. In 1996, two families with deletions encompassing *ELN* and *LIMK1* were evaluated and the individuals presented with supravalvular aortic stenosis and the characteristic cognitive profile of WBS patients, including poor spatial construction skills and proficient verbal ability [196]. However, a later study reported two individuals with the same partial deletion who did not present the WSCP [197, 198].

The *Limk1* knockout model presented significant abnormalities in spine morphology and synaptic function and presented altered fear response and spatial learning. The authors conclude that *Limk1* plays a critical role in the morphogenesis and function of dendritic spines [199].

Therefore, it seems that although *LIMK1* might contribute to the visual spatial constructive cognition, its haploinsufficiency is not sufficient and other genes in the WBS region might be involved.

3.5.3 CAP-GLY Domain containing linker protein 2

The CAP-GLY domain containing linker protein 2 (*CLIP2*, OMIM 603432) regulates cytoskeleton by polymerization of the microtubule network [200, 201]. It is abundantly expressed in dendrites and cell bodies of neurons in the brain [202].

Its role in the craniofacial dysmorphisms and neurocognitive profile in WBS patients is controversial. Patients with smaller atypical deletions including *CLIP2,* but not *GTF2I* or *GTF2IRD1,* presented a milder phenotype with visual spatial impairment, a higher verbal score compared to performance score with a normal intellectual quotient [147, 184]. It was proposed that *CLIP2* haploinsufficiency could be associated with the neurocognitive profile. However, other patients with partial deletions involving *CLIP2* did not present the WSCP [144, 203], including a pure hemizygous *CLIP2* deletion in two unaffected siblings [204].

The homozygous and heterozygous mouse model of *Clip2* present mild growth deficiency, brain abnormalities, hippocampal dysfunction and specific deficits in motor coordination [205].

As in the case of *LIMK1*, it seems that *CLIP2* haploinsufficiency is not the sole cause of the phenotype but that it might contribute to the visual spatial and cognitive impairments along with other genes present in the region.

3.5.4 Bromodomain adjacent to zinc finger domain 1B

Bromodomain adjacent to zinc finger domain IB (*BAZ1B*, OMIM 605681) is a subunit of a chromatin-remodeling complexes, involved in DNA repair [206]. *BAZ1B* is highly expressed in cranial neural crest derived mesenchyme [207].

Murine models showed that *BAZ1B* could be a potential candidate gene involved in craniofacial phenotype of WBS. Random mutagenesis in mice resulted in a heterozygous L733R change in a highly conserved amino acid [207]. Homozygous mice were smaller and had widened bulbous foreheads, shortened snouts and a reduction in parietal and nasal bone [207]. Heterozygous mice also presented similar alterations, including a decrease in cranium width to height ratio and a decrease in palatine bone [207]. Partial deletion mouse model including *Baz1B* also presents with an abnormal craniofacial appearance [162]

3.5.5 Frizzled 9

Frizzled 9 gene (*FZD9*, OMIM 601766), first identified in 1997 [208], is selectively expressed throughout life in the hippocampus [209] and it has been postulated that it may act as a Wnt receptor in the canonical Wnt pathway, signaling via β-catenin pathway [210].

Frizzled 9 could not be established as a potential candidate involved in the WBS specific neurocognitive profile, since patients with smaller atypical deletions excluding *BAZ1B* and *FZD9* presented with milder

facial features and moderate neuropsychological deficits [150]. However, the frizzled 9 murine model pointed towards its potential involvement in the neurocognitive phenotype. Null and heterozygous mice presented with increased apoptotic cell death and increased proliferation of precursors during hippocampal development [211]. Null mice presented severe deficits in learning and memory, which could reflect hippocampus functional deficits [211]. Heterozygous mice presented milder alterations in spatial memory and lower seizure threshold [211]. A second murine model for *Fzd9* was analyzed and it did not exhibit the same cognitive alterations, instead it had an abnormal B cell development [212]. Further studies of this model revealed that frizzled 9 null mice also presented low bone mass caused by impaired bone formation, with defects in matrix mineralization [213].

3.5.6 Syntaxin 1A

Syntaxin 1A (*STX1A*, OMIM 186590) encodes a protein that belongs to the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) complex [214] which is involved in vesicle fusion process [215]. The SNARE complex plays a major role in insulin exocytosis, as well as in neurotransmitter release.

Stx1A hemizygous mice did not present any apparent behavioral or cognitive phenotype [216]. Homozygous mice for a truncated form of *Stx1a* presented altered synaptic plasticity [217] and both, homozygous and heterozygous mice, showed abnormal behavior similar to neuropsychological alterations observed in psychiatric patients [218].

3.5.7 Eukaryotic translation initiation factor 4

The eukaryotic translation initiation factor 4 (*EIF4H*, OMIM 603431) gene encodes a factor involved in the initiation phase of protein synthesis [219]. Protein translation is involved in synaptic plasticity and it has also been associated with autism [220, 221]. The role of *EIF4H* in the cognitive profile of WBS patients has not been studied. However, the single gene knockout model presented central nervous

system morphological alterations, including smaller brain volume and altered brain morphology [222]. Histological analysis revealed a reduction in the number and complexity of neurons. Behavioral analysis also showed severe impairments memory formation and fear-related associative learning [222]. Further research is needed to elucidate the potential role of *EIF4H* in WBS cognitive profile.

3.6 Metabolic phenotype

The metabolic phenotype, specifically alterations in glucose metabolism, observed in individuals with WBS have been associated with two genes, the Mlx-interacting protein like and the syntaxin 1A.

3.6.1 Mlx-interacting protein like

Mlx-interacting protein like (*MLXIPL*, OMIM 605678), also known as carbohydrate response element binding protein (ChREBP), encodes for a member of the basic helix-loop-helix / leucine zipper family of transcription factors and forms heterodimers with the Mlx protein to bind the carbohydrate response element (ChoRe). Target genes of ChREBP are involved in gluconeogenesis, lipogenesis, glycolysis and the NAPDH supply system [159]. It is ubiquitously expressed, but more highly expressed in the liver, brown and white adipose tissue, small intestine, kidney and muscle [159].

The single gene knockout model presented increased plasma glucose and insulin levels when fed with standard diet, decreased lipogenesis, decreased ability to metabolize glucose leading to accumulation of glycogen content in the liver and a decrease in adipose tissue [159]. Heterozygous mice were not studied.

Genome wide association studies (GWAS) further support a role of *MLXIPL* in WBS metabolic phenotype. Four single nucleotide polymorphisms (SNPs) in the *MLXIPL* region were associated with increased triglyceride levels [223].

3.6.2 Syntaxin 1A

Syntaxin IA has also been associated with the impaired glucose metabolism observed in WBS patients. Glucose-stimulated insulin release has been shown to follow a biphasic pattern in vitro and in vivo studies [224]. The presence of glucose in the β-cells of the pancreas provokes a rapidly initiated and transient first phase followed by a sustained second phase[225]. The SNARE complex, in particular STX1-A protein, binds with calcium and potassium channels involved in the exocytosis of docked and primed insulin granules [226].

GK rats, which are used as a model for type 2 diabetes, had marked reductions in the expression of *Stx1a* [227]. Several mouse models have been created to study the consequences of altered STX1A levels in glucose metabolism [216, 228, 229]. A transgenic mouse overexpressing *Stx1a* presented a slight decrease in insulin exocytosis that proved sufficient to provoke glucose intolerance and male mice also presented insulin intolerance [228]. The *Stx1a* knockout model had marked reduction in the first phase of insulin release, without changes in the second phase, impaired oral glucose tolerance and low serum insulin levels [229]. This suggests that *STX1A* plays an important role in glucose homeostasis, specifically in the docking and fusion of insulin granules during the first phase of insulin release [229].

Further evidence supporting the involvement of *STX1A* in glucose deregulation observed in WBS patients, are SNPs in the gene associated with earlier age of onset and higher daily insulin requirements in individuals with type 2 diabetes or with protection from impaired glucose regulation in a cohort of overweight individuals [230, 231].

OBJECTIVES

OBJECTIVES

The global aims of this thesis project were to improve the definition of the Williams-Beuren syndrome phenotype, the natural history, characterize the deletion and the molecular mechanisms involved, and identify possible candidate genes and clinical-molecular correlations.

To achieve these global objectives, we established the following specific objectives:

1. Characterize the 7q11.23 deletion to describe in detail the breakpoint location and identify hotspots by the analysis of site-specific nucleotides and PCR amplification of junction fragments. Identify possible *cis* and *trans*-acting mechanisms involved in Williams-Beuren syndrome deletion by the sequence analysis of hotspots and candidate gene.
2. Characterize novel and previously identified metabolic phenotypes in Williams-Beuren syndrome by the analysis of a complete clinical, biochemical and molecular database of patients with William-Beuren syndrome and explore the association of metabolic phenotype with putative candidate genes.
3. Contribute to the better definition of the cardiovascular disease in Williams-Beuren syndrome patients using the analyses of a complete database of patients with William-Beuren syndrome and specific diagnostic procedures, including echocardiography, ambulatory blood pressure monitoring and CT angiogram, and explore clinical and clinical-molecular correlations associated with the cardiovascular phenotype, including an association analysis with possible genetic modifying factors.

CHAPTER 1

Hotspots for recurrent meiotic rearrangements mediated by segmental duplications at 7q11.23

María Gabriela Palacios Verdú, Núria Rivera, Raquel Flores, Alejandro Cáceres, Anna Antonell, Juan Ramón González, Ivon Cuscó, Luis Alberto Pérez-Jurado

In preparation

WBS is a recurrent genomic disorder caused by the deletion of 1.55-1.83 Mb at chromosomal band 7q11.23. WBS deletion is generated by non-allelic homologous recombination between misaligned blocks. In this study we performed a fine molecular characterization of the de novo submicroscopic deletions in a cohort of 720 unrelated patients with a *de novo* 7q11.23 deletion.

Approximately 86.4% of cases arise due to crossing over events between misaligned B-blocks generating a 1.55 Mb deletion. The majority of these deletions are located in the proximal half of the B-block, before *NCF1* gene. Moreover, 33% of the deletions occur at a previously defined hotspot within *GTF2I* almost exclusive of paternal-origin deletions (7.5-fold increased compared to the remaining ≈100 kb). Approximately 22% of deletions are mediated by a paracentric inversion. Two hotspots located within *GTF2IRD2* have been found in 16% and 39% of patients. The remaining 12% the deletions occur due to crossing over events between misaligned A-blocks with a size of 1.83 Mb.

Putative *cis*-acting mechanisms that can influence the rate of recombination at the three described hotspots include higher GC content, higher frequency of *Alu* repeats and recombination motifs. *PRDM9* gene, a putative *trans*-acting factor, was not associated with WBS deletion.

Hotspots for recurrent meiotic rearrangements mediated by segmental duplications at 7q11.23

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ABSTRACT

The frequent sporadic occurrence of Williams-Beuren syndrome (WBS) indicates a high rate of *de novo* deletion formation at 7q11.23, ~10^{-4} per gamete per generation. Most WBS deletions arise from non-allelic homologous recombination (NAHR) between specific blocks of highly homologous region-specific segmental duplications (SDs), and may be facilitated by heterozygosity for paracentric inversions and other structural variants. We have characterized the deletion at the 7q11.23 WBS locus in a large cohort of 720 unrelated patients, including atypical (12/720, 1,6%) and recurrent deletions of 1,55Mb (86,4%) or 1,83Mb size (12%). By dosage analyses of site-specific nucleotides or *cis-morphisms* in trios, we have defined the region of chromosomal exchange in a subset of 177 *de novo* recurrent 1,55Mb deletions. A previously defined NAHR hotspot has been narrowed-down to a 1,4 kb region of complete sequence identity, showing clear bias in favour of paternal and intrachromosomal origin. Interchromosomal exchange in inversion carriers (25% of transmitters) also occurred on specific hotspots with some parental bias. Rearranged chromosomes resulted in different copy number of the *NCF1* gene, as well as the generation of chimeric *GTF2IRD2* transcripts in a subset of probands. *Alu* repeats and other putative recombination stimulating sequences and DNA structures are located within and around the hotspots. However, we did not find evidence of any contribution of *PRDM9* alleles as *trans*-acting factors responsible for different NAHR rate in deletion transmitters. Therefore, NAHR between SDs does not occur randomly; instead, several parent-of-origin-hotspots for positional preference of recombination can be identified within the SDs.

INTRODUCTION

Williams-Beuren syndrome (WBS, OMIM 194050) is a developmental neurocognitive disorder with multi-systemic manifestations of variable expressivity caused by a heterozygous deletion at chromosomal band 7q11.23. Although there are a few cases of vertical transmission of WBS as an autosomal dominant trait, it mostly occurs sporadically with an estimated prevalence of 1/7,500 among newborns (Strømme et al. 2002). Therefore, the rate of *de novo* deletion formation at 7q11.23 in the human population is very high, around 10^{-4} per gamete per generation.

The 7q11.23 chromosomal region has a complex genomic architecture that can predispose to chromatid mispairing. Three large region-specific segmental duplications SDs (centromeric, medial and telomeric), composed of three differentiated blocks each, called A, B and C, flank the WBS common deletion region (Peoples et al. 2000; Valero et al. 2000). Unequal meiotic crossing-over between misaligned blocks located in the same orientation within the centromeric and medial SDs, either block B or block A, has been shown to be the molecular mechanism for the recurrent WBS deletions (Pérez Jurado et al. 1996; Urbán et al. 1996; Bayés et al. 2003). The great majority of deletions occur due to non-allelic homologous recombination (NAHR) events between the centromeric and medial B-blocks generating a deletion of 1.55 Mb, while 5-10% arise due to NAHR between misaligned centromeric and medial A-blocks with a size of 1.83 Mb (Bayés et al. 2003).

The presence of structural variation in the region can further predispose heterozygous carriers to chromosomal misalignment in meiosis and subsequent NAHR. Inversion polymorphisms have been associated with an increased risk of meiotic rearrangements leading to several recurrent genomic disorders (Gimelli et al. 2003; Koolen et al. 2008). At 7q11.23, inversion-mediated deletions have been reported to account for approximately 25% of patients, while large copy number variants (CNVs) at the flanking regions are also present in 5% of transmitting parents (Osbore et al. 2001; Bayés et al. 2003; Cuscó et al. 2008).

It has been proposed that the rate of recurrent rearrangements is directly proportional to the length and the degree of nucleotide homology between SDs and inversely related to the distance between them (Turner et al. 2008). Several studies have analyzed other factors that can influence the rate of rearrangement formation. The breakpoint regions of CNVs were

analyzed and the authors found an enrichment of secondary structures such as G-quadruplexes, cruciforms, recombination motifs, *Alu* signal recognition motifs and microsatellites (Watson et al. 2014; Conrad et al. 2010). A recent publication, identified and analyzed 8,943 deletion breakpoints in 1,092 samples from the 1000 Genomes Project and found that NAHR breakpoints were associated with high GC-content, higher density of CpG motifs, open chromatin with higher DNA accessibility and active histone marks (Abyzov et al. 2015). Interestingly, this demonstrates that NAHR events can also occur in germ cells or in early embryonic cells without replicating DNA (Abyzov et al. 2015). We also reported a significant sex bias in favor of paternal origin at a 3.4 kb hotspot for the WBS 1.55 Mb deletions in a small cohort of patients (Bayés et al. 2003).

The recombination rates can also be influenced by *trans-acting* mechanisms. In recombination hotspots it was observed an enrichment of a 13-mer motif (5´-CCNCCNTNNCCNC-3´) in approximately 40% of the identified autosomal human hotspots (Myers et al. 2008). This motif is recognized by the PR domain containing protein 9 (*PRDM9,* OMIM 609760) (Berg et al. 2010). *PRDM9* allelic variations has also been implicated in an increased frequency of NAHR rates in genomic disorders (Berg et al. 2010; Borel et al. 2012).

Here, we report the our molecular analysis of 720 unrelated patients with a *de novo* submicroscopic deletion at the WBS locus on 7q11.23, including the detailed analysis of breakpoints in 177 cases with the most common types of rearrangements. The molecular characterization of the WBS deletion can contribute to increase the knowledge of recurrent genomic disorders mediated by NAHR and putative *cis* and *trans*-acting factors involved.

RESULTS

Size and parental origin of the 7q11.23 deletions in patients with WBS

Atypical deletions were present in 1.6% (12/720) patients in our cohort. Nine cases had larger deletions, four had a low-recurrent 2 or 4 Mb deletion extending towards the telomere, four had non-recurrent deletions of 8 and 10 Mb size and an atypical deletion of 1.7 Mb extending to the centromere. The remaining three cases had smaller atypical deletions that have been previously characterized and reported (Antonell et al. 2010a; Delgado et al. 2013). The great majority of patients (86.4%, 622/720) were identified as carrying 1.55 Mb deletions caused by NAHR

between blocks B, whereas 12% of cases (87/720) had 1.83 Mb deletions mediated by blocks A. Complete trios were available from 402 families. Deletions were *de novo* in all cases, of maternal origin in 51% (205/402) and paternal origin in 49% (197/402). There was no significant parental-origin bias associated with the deletions (p=0.488). Given the complex structure of the blocks A of SDs, composed of smaller segmental duplications located on the same region and elsewhere on chromosome 7 (up to 17 copies), we focused on the analysis of the junction fragment in 177 patients with the most common 1,55 deletion mediated by blocks B, which are present in three copies on the reference genome.

Breakpoint mapping in 1.55 Mb deletions

Quantification of multiple site-specific nucleotides (SSN) located throughout the block B was obtained to narrow down the location of the strand exchange in individuals with 1.55 Mb deletions (Figure 1). Approximately 76.3% (135/177) of the deletions occur in the proximal half of the B-block containing *GTF2I/GTF2IP* and *NCF1/NCF1P1*. In 54.2% (77/142) the breakpoint was suggested to occur before *NCF1,* since individuals had 2 or more *NCF1* functional copies. We further refined the breakpoint by genotyping SSN2 located within *GTF2I* (exon 21) and 45.5% (35/77) were located between exon 16 and 21 of *GTF2I* (SSN1 and SSN2)(Figure 1).

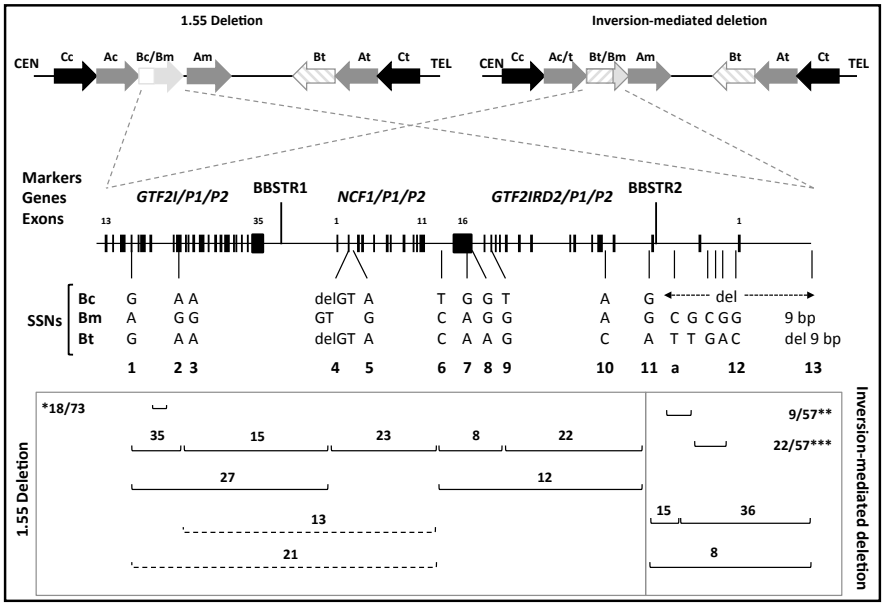

Figure 1. Schematic representation of SSNs distributed along the B-block and the frequency of deletion at each interval for 1.55 Mb deletion (n=177) and inversion-mediated deletion (n=59) (Asterisks mark each hotspot: * JF-GTF2I, ** JFINV1, ***JFINV2). Exons are depicted as black boxes and gene symbols are on top. The black bars delineate the interval in which the recombination event occurred and above the number of patients at each location.

In 16% (23/142) the breakpoint was located at *NCF1,* patients had only one functional copy. For the remaining deletions with breakpoints located before *GTF2IRD2* we were partially able to define the breakpoint location (Figure 1). In 7.3% (13/177) the deletion was located after SSN2, but due to gene-conversion events in the progenitors we were unable to conclude if *NCF1* was also involved. In 11.9% (21/177) the deletion occurred before *GTF2IRD2* but due to gene-conversion events and technical problems we could not further refine the breakpoint.

Approximately 24% (42/177) of the deletions occurred within *GTF2IRD2*. We were able to refine the breakpoint location in 71% (30/42); of which 27% (8/30) were located between exon 14 and 16 and the remaining 73% (22/30) were located between exon 14 and intron 3 of *GTF2IRD2*. Breakpoints at this region generate a chimerical copy, with the first exons belonging to the medial copy and the last to the centromeric copy.

Approximately 7.5% (20/265) of individuals had more than two functional copies of *NCF1* and approximately 26.7% (27/101) had two functional copies secondary to gene-conversion events when deletions were located at *GTF2IRD2* or mediated by an inversion.

The frequency of parental origin was compared with respect to deletions occurring before and after *NCF1* and found that deletions with a breakpoint before *NCF1* were significantly more frequent of paternal origin (61%, 47/77) compared with deletions occurring after *NCF1* (29%, 19/65) ($\chi^2(1, n=142)=14.34$, $p=0.0002$).

We previously described a 3.4 kb hotspot located within *GTF2I* for 1.55 Mb deletion (JF-GTF2I) (Figure 2)(Bayés et al. 2003). We have increased our sample size by studying JF-GTF2I in 73 cases with breakpoints suggested to occur before SSN2. We were able to successfully amplify the internal control for 75% (55/73) of individuals, in 18 samples the amplification of both the fragment and the internal control failed likely due to poor DNA integrity. The specific 3.4 kb amplification product was obtained in 43.6% (24/55) individuals, whereas the remaining

56.4% (31/55) cases were found negative. In 16 out of the 24 cases with amplification, both parents were negative for the 3.4 kb amplification product, indicating that the fragment that specifically amplified in the patient corresponds to a *de novo* recombinant Bc/Bm block junction fragment. In the remaining 8 patients, the same amplification product was obtained in one or both progenitors indicating the presence of *trans-morphisms* or lack of copy specificity at the block-specific nucleotide-sites. Thus, one primer might anneal not specifically, becoming complementary to the same block B than the other primer, and giving rise to the same 3.4-kb fragment (false positive). In order to clarify these cases, PCR products from the recombinant WBS-Block B were purified from agarose gels and sequenced to identify the crossover as evidenced by the transition from Bc to Bm using specific paralogous sequence variants (PSVs). In 2 of the 5 ambiguous cases, sequencing permitted to distinguish a *de novo* junction fragment (true positive) compared to a possible polymorphic variant (false positive) inherited from the progenitors. In 2 cases we confirmed that the amplified product was a false positive (*trans-morphism*) and in 2 cases we were unable to sequence the amplified product. It is worth mentioning that although true negative results for the 3.4 kb fragment amplification excludes the presence of the breakpoint at this interval, the putative location of their breakpoints can still be located within the 12 kb cluster, probably flanking the 3.4 kb interval.

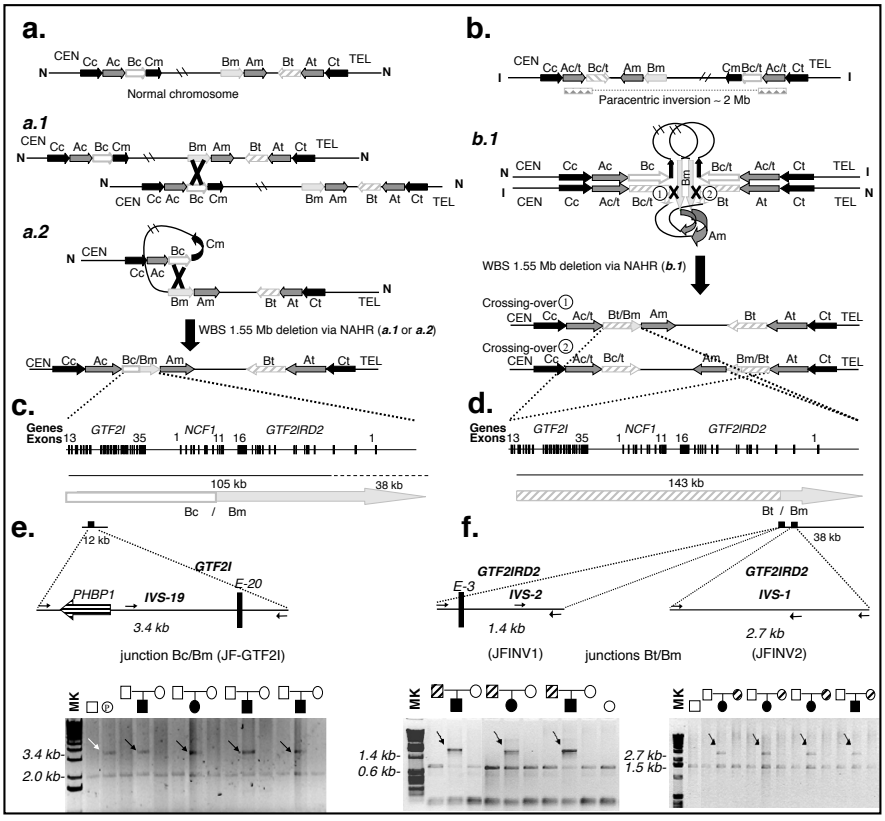

Figure 2. Schematic representation of the architectural structure in **a.** normal and **b.** inverted 7q11.23 genomic region. Models for unequal crossover events resulting in WBS chromosomes with common 1.55 Mb deletion and PCR amplification of junction fragments. Using an scheme with the large blocks of LCRs represented in black (Cc, Cm and Ct), dark grey (Ac, Am and At) and light grey (open: Bc; filled: Bm and diagonal stripped: Bt) arrows, the different mechanistic models for deletions are shown: **a1**. Interchromosomal rearrangement, **a2**. Intrachromosomal rearrangement and **b1.** Interchromosomal rearrangement in inversion carriers. Below each rearrangement, the resulting WBS deleted chromosomes with the specific recombinant blocks B are represented. **c, d.** Representation of the entire ~143 kb length of block B with its gene content (exons depicted as blackened boxes with gene symbols on top). Note that the last 38 kb (dotted line) are absent in block Bc. In **e,f,** detection of patient-specific junction fragments. **e.** Specific primers for block Bc and Bm/Bt allow the amplification of a recombinant Bc-Bm fragment of 3.4 kb spanning introns 19-20 of the *GTF2I* gene. A nested primer that anneals to all three blocks and amplifies a 2.0 kb product from Bm and Bt was used as an internal positive PCR control. **f.** Two different PCR assays with block-specific primers amplify the novel recombinant Bt-Bm fragment in inversion-mediated WBS deletions, also including internal PCR positive controls (1.4 and 2.6 kb bands). Two *de novo* junction fragments were identified, located in intron 2 (1.4 kb) and intron 1 (2.6 kb) of the *GTF2IRD2* gene, respectively. Diagonally striped symbols represent

carriers of the 7q11.23 paracentric inversion, transmitting the rearranged chromosomes.

When the presence of JF-GTF2I breakpoint was compared with the parental origin of the deletion, we observed that JF-GTF2I occurs almost exclusively in deletions of paternal (17/124, 13.7%) than maternal origin (1/141, 0.7%) (χ^2 (1, n=265)=17.61, p<0.0001).

Generation of *GTF2IRD2-P1* chimeric transcripts

To further document the junction fragment within *GTF2IRD2,* we investigated whether chimeric transcripts between the medial and centromeric copies of the gene were expressed. A fragment from exon 11 to exon 16 was amplified from cDNA of LCL of WBS patients to analyze centromeric PSVs. As observed in Figure 3c, patients predicted by SSN analysis to harbor the chimera were heterozygous for centromeric specific changes. Except for one patient with an inversion-mediated deletion, none of the controls or patients with other molecular variants of *GTF2IRD2* had centromeric changes.

We decided to clone a larger fragment of *GTF2IRD2* (from exon 11 to the end of exon 16) and sequence it to corroborate our previous findings. As observed in Figure 3d, we were able to analyze 14 changes in exon 16 and a change in exon 14, including the loss of the stop codon in exon 16 (hexagon). All changes in exon 16 (including the stop-loss) and the first change of exon 14 corresponded to the centromeric copy.

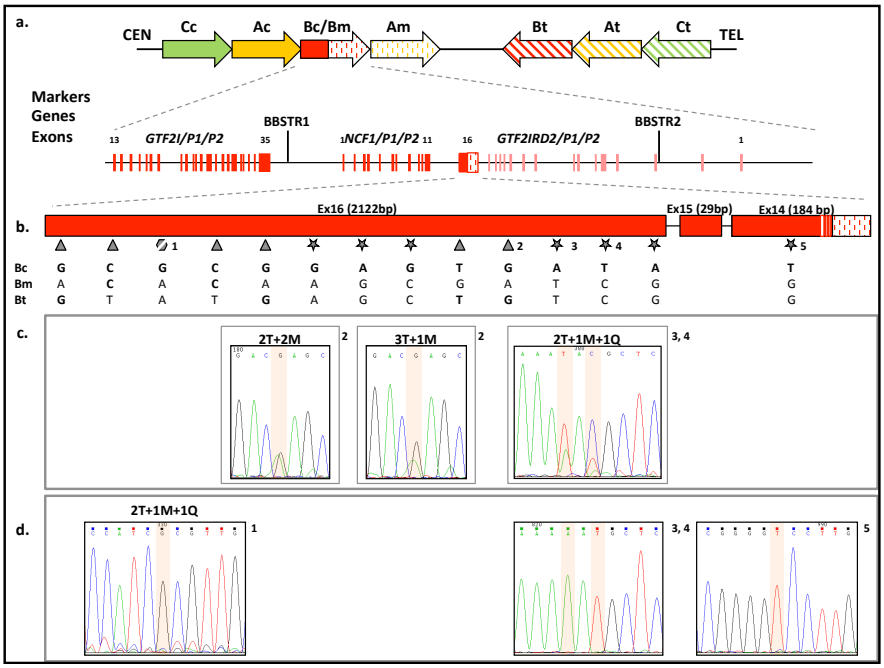

Figure 3. Schematic representation of the architectural structure of **a.** deletion of 1.55 Mb with an unequal crossover between Bc and Bm. The large blocks of LCRs are represented by green (C-blocks), yellow (A-blocks) and red (B-blocks). Solid patterns correspond to the centromeric copy, dotted pattern for the middle copy and diagonally striped for the telomeric copy. Exons are depicted in red boxes with gene symbols on top. **b.** Representation of exons 14 to 16 with the paralogous sequence variants (PSVs) beneath. Triangles represent PSVs that discriminate the telomere from the centromeric and medial copy, stars represent PSVs that discriminate the centromeric from the telomeric and medial copy, and the hexagon represents the stop-loss specific of the centromeric copy. The number beside each PSV corresponds to the genotyped PSVs **(c, d)**. Striped lines at exon 14 indicate that the crossover event could have occurred at any region after the last genotyped PSV. **c.** Sequenced PSVs from cDNA of patients with different *GTF2IRD2* molecular variants. Genotyped PSV of *GTF2IRD2* molecular variants (2T+2M and 3T+1M) show that the telomeric PSV is expressed. PSVs shown for 2T+1M+1Q variant discriminate centromeric blocks. **d.** Sanger-sequencing of a subclone of the chymeric copy of *GTF2IRD2* (2T+1M+1Q) indicating the stop-loss at exon 16 and three PSVs at the beginning of exon 16 and exon 14.

Inversion-mediated deletion and hostpot detection

In order to determine the frequency of inversion-mediated deletions in patients with 1.55 Mb WBS deletions, we analyzed SSN11 within the *GTF2IRD2* gene (Bayés et al. 2003). At these positions close to the end of the ~105-kb alignment between Bm and Bt, there should

always be a gain of a Bt-type sequence and loss of a Bm-type sequence if the rearranged WBS chromosome was originated by an inter-chromosomal unequal exchange in an inversion carrier, as we previously predicted (Figure 2)(Bayés et al. 2003). We identified that 59 of 265 deletions occurred through this mechanism, implicating that at least 22.2% of the transmitting progenitors are carriers of the inversion polymorphism. There was no significant association between parental origin and deletions mediated by the inversion polymorphism.

We confirmed the heterozygosity for the inversion polymorphism in all tested individuals (7/7) by three-color FISH in interphase nuclei whose carrier status had been predicted by SSN-typing.

To more precisely refine the sites of crossover in our series of WBS patients with inversion-mediated deletion an additional SSN was genotyped (SSNs 11a). The deletion was located between SSN11 and 11a in 29.4% (15/51), the remaining 70.6% (36/51) were located after SSN11A. In 13.6% (8/59) cases we were unable to refine the location of the breakpoint due to technical complications (Figure 1).

We have designed a PCR based analysis to amplify two novel putative junction fragments of inversion-mediated deletions located between intron 1 and 3 of *GTF2IRD2* (Figure 2). We studied the proximal and distal inversion junction fragment in 57/59 (96.6%) of the inversion-mediated deletions. In 2 individuals, due to poor DNA quality we were unable to amplify the control band. The proximal 1.4 kb junction fragment (JFINV1) was amplified in 22% (13/59) samples. In 7 cases, the amplification was obtained only for the patient confirming the presence of a *de novo* junction fragment. In the other 6 cases, we obtained the amplification product also for one or both parental samples. We confirmed the presence of a *de novo* junction fragment in 2 of the 6 cases by sequencing the obtained product. In 4 cases we confirmed the presence of a *trans-morphism.*

We were able to amplify the distal 2.6 kb junction fragment (JFINV2) in 28 of the 57 individuals. In 20 individuals, the amplification was obtained only for the patient confirming the presence of a *de novo* junction fragment. In 5 cases, we obtained the amplification product for one or both parental samples. We confirmed the presence of a *de novo* junction fragment in 2 cases by sequencing the obtained product, in 3 cases the amplification product confirmed the presence of a *trans-morphism* and in one case we were unable to observe the PSV.

We did not find a statistical significant association with parental origin of the deletion for JFINV1 or JFINV2. However, deletions at JFINV1 were more frequent of paternal origin (6/9) and deletions at JFINV2 were more frequent of paternal origin (15/22), (χ^2 (1, n=59)=2.14, p=0.0144).

Cis-acting mechanisms in NAHR: Sequence analysis at breakpoints

The frequency of sequence motifs associated with recombination and replication is in concordance with the distribution along the entire block B, except for the immunoglobin heavy chain class switch repeat (5´-GGGGT-3´) that shows a 2.9, 8.4 and 5.9 fold-increase within JF-GTF2I, JFINV1 and JFINV2 hotspots, respectively. At the JF-GTF2I hotspot we also found the DNA polymerase α/β frameshift motif (5´-ACCCCA-3´) increased 14.4 fold, whereas the autonomously replicating sequence (ARS) consensus from *Schizosaccharomyces pombe* (5´-WRTTTATTTAW-3¨) appears to be increased 10.5 fold only in the JFINV1 hotspot. Interestingly, fold-changes ranging between 4 and 8 were also found for the immunoglobulin heavy chain class switch repeat and the DNA polymerase α/β frameshift motifs in the flanking regions of all WBS hotspots. *PRDM9* motif (5´-CCNCCNTNNCCNC-3´)(Berg et al. 2010) and its complementary sequence were searched in the three hotspot regions and in JFINV2 and JF-GTF2I a 3.6 and 2.8- fold-enrichment was found when compared to the block B, respectively.

RepeatMasker was run to calculate the total percentage of interspersed repeats and the GC content in the region. Whereas the GC content showed an uniform distribution along the entire block B (47%), a higher density of repetitive elements were found in the last 38 kb of the block B (50% in the first 103 kb and 70% in the last 38 kb). We also found a significantly higher density of *Alu* repeats (types Sx/Jb/Y) inside the three defined hotspots, encompassing 91.7% of JFINV1, 69% of JFINV2 and 63.2% of JFGTF2I, respectively.

To further investigate the characteristics of the sequences showing positional preference for strand exchange in WBS, we compared them among themselves and with all other reported NAHR hotspots associated with genomic disorders using the BLAST algorithms. The only significant stretch of sequence identity was among all three WBS hotspots and the LCR17pD/A alternative hotspot associated with the large SMS deletions in 17p11.2 (Shaw et

al. 2004). The detected region with sequence homology corresponds to the 26-bp core of an *Alu* Sq/x element (5'-CCTGTAATCCCAGCACTTTGGGAGGC-3') (Kariya et al. 1987).

Trans-acting mechanisms in NAHR: *PRDM9* Genotype

The frequencies of *PRDM9* alleles in our cohort (n=329) were 0.921 for the A-allele and 0.079 for the non-A alleles (C: 0.044, D: 0.015, L8: 0.018 and L15 / L16: 0.0015) (Table 1).

PRDM9 Alleles	Nº Reps Digested	Inversion-mediated (n=62)	No Inversion mediated (n=240)	JF-GTF2I (n=24)	All Transmitters (n=322)	Non-transmitters (n=336)	EU Frequency	Spanish Frequency
A / B	13 Reps Digested	0.935 (58/62)	0.929 (223/240)	0.958 (23/24)	0,929 (299/322)	0,914 (307/336)	0,875	0.744
C	14 Reps Digested	0.048 (3/62)	0.042 (10/240)	0,000 (0/24)	0,043 (14/322)	0,045 (15/336)	0,01	0.013
D	14 Reps Not digested	0,000 (0/62)	0,000 (0/240)	0,000 (0/24)	0,000 (0/322)	0,030 (10/336)	0,01	0.02
L8	15 Reps Digested	0.016 (1/62)	0.025 (6/240)	0.042 (1/24)	0,003 (8/322)	0,012 (4/336)	0,003	0.021
L15/L16	13 Reps Not digested	0,000 (0/62)	0.004 (2/240)	0,000 (0/24)	0,025 (1/322)	0,000 (0/336)	0,000	0.004

Table 1. *PRDM9* allelic frequencies in the transmitter, non-transmitter group and in Europeans (Berg et al. 2010; Alemany-Schmidt et al. 2014).

No significant differences were found when comparing allelic and genotypic frequencies between the transmitting and the non-transmitting group (Table 1). However, the D-allele (an allele with 13 repeats that is digested by *Bst*UI) was only found in the non-transmitter group (allelic frequency of 0.030, p=0.002). We also calculated the allelic frequency for the hotspot JF-GTF2I and searched if there was any relationship with the presence of an inversion or the size of the deletion. As seen on Table 1, there was a slight increase of the allele with 15 repeats digested with *Bst*UI in the JF-GTF2I compared to all the other groups but it did not reach statistical significance.

Inversion Haplotype Analysis

To establish the prevalence of the inversion polymorphism in the general population we performed three color FISH in interphase nuclei from 48 controls, including

25 non-transmitting WBS parents. Only two individuals were found heterozygous for the inversion polymorphism, which gives a population frequency of 4.2% (95% confidence interval 0-8.8%), which is significantly different from transmitting progenitors (χ^2 (1, n=512)=8.53, p=0.003).

In order to investigate whether most inversions had originated in a common ancestor or through recurrent genomic mutations, we determined the haplotypes associated with the inversion polymorphism using siblings and/or grandparents to establish the phase (n=8). There was not a single haplotype shared by the eight chromosomes identified with the inversion (not shown). Since chromosomal inversions are known to suppress recombination in heterozygous carriers, our data suggest that most microinversions in 7q11.23 occurred through independent mutational events in multiple founders.

We studied further the haplotypes associated with the inversion by genotyping five SNPs predicted to infer the inversion (rs1109512, rs2528997, rs4717820, rs6460068) and rs799160, also located in WBS region, by Sequenom technology (Figure 4).

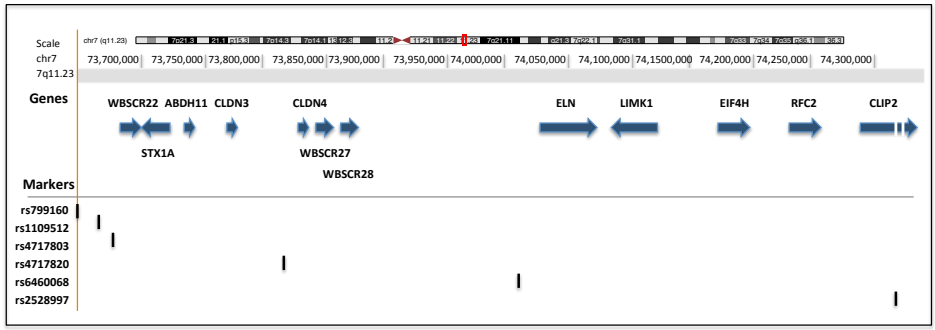

Figure 4. Genomic location of the six genotyped SNPs at 7q11.23 with respect to the gene content in the region (labeled in black below the ideogram of chromosome 7).

The allelic frequencies of three SNPs (rs2528997, rs4717820 and rs799160) were significantly different between the inversion and no-inversion carriers groups (p=0.004, p>0.0001 and p=0.0032, respectively) (Table 2). The haplotype of individuals homozygous for the not inverted allele were constructed from WBS patients. The allelic, genotypic and haplotype frequencies were compared between the inversion and non-inversion carriers groups (Table 2). After comparing with this new haplotypes, the allelic

frequencies were significantly different in the same three SNPs, as shown in Table 2. There were statistically significant differences in the genotypic frequencies of rs2528997, rs4717820 and rs799160 (p=0.001, p<0.001 and p<0.001, respectively) between both groups, with an increase number of heterozygous individuals in the inversion-carriers group.

SNP	Alleles	HapMap (CEU) Freq	Non-inversion carriers Freq (2730 alleles)	Inversion carriers Freq (142 alleles)	HWE	$\chi 2$ (p-value)	Odds Ratio (95%CI) (p-value)
rs1109512	C	0.300	0.277 (755)	0.225 (32)	0.49	1.77 (0.180)	0.67 (0.44-1.03) 6.80e-02
	G	0.700	0.723 (1975)	0.775 (110)			
rs2528997	T	0.258	0.293 (800)	0.444 (63)	0.47	6.290* (0.0121)	1.64 (1.15-2.34) 6.70e-03*
	C	0.742	0.707 (1930)	0.556 (79)			
rs4717803	A	0.310	0.277 (755)	0.225 (32)	0.49	1.770 (0.1834)	0.67 (0.44-1.03) 6.80e-02
	G	0.690	0.723 (1975)	0.775 (110)			
rs4717820	G	0.210	0.297 (811)	0.556 (79)	0.091	40.92* (<0.0001)	2.65 (1.83-3.83) 0.00e+00*
	A	0.790	0.703 (1919)	0.444 (63)			
rs6460068	T	0.434	0.475 (1298)	0.415 (59)	0.81	1.76 (0.1876)	0.94 (0.66-1.33) 7.13e-01
	C	0.566	0.525 (1432)	0.585 93)			
rs799160	A	0.469	0.511 (1394)	0.669 (95)	0.0026*	17.319* (<0.001)	0.56 (0.38-0.83) 4.20e-03*
	G	0.531	0.489 (1336)	0.331 (47)			

Table 2. Allelic frequency of SNPs located in 7q11.23 in CEU HapMap, no-inversion carriers and inversion carriers (asterisks denote statistical significance).

The OR was calculated for each SNP (Table 2), with significant results for rs2528997 (OR=1.64, CI95: 1.15-2.34, p=6.70e-03), rs4717820 (OR=2.65, CI95: 1.83-3.83, p=0.00e+00) and rs799160 (OR=0.56, CI95: 0.38-0.83, p=4.20e-03). Two SNPs, rs2528997 and rs799160, remain statistical significant after Bonferroni´s correction (p<0.008).

Haplotype analyses were performed, and the frequencies were compared between inversion carriers and non-carriers. There were five haplotypes that were at least three times more frequent in the inversion carriers compared to the non-inversion carriers (GTGACG, GTGGCA, CCAGTA, CCAGCG and GCGGCA).

Multidimensional method and prediction method based in odds

ratio (OR) were used to calculate the predictability of these SNPs to infer the inversion. As shown in Figure 5A, by the multidimensional approach the predictability was of 63%, with a sensibility of 73% and specificity of 61%. With the OR based method, two haplotypes were associated with the inversion (GTGGCA and GCGGTA) and were present in approximately 20% of inversion-carriers. These haplotypes had a predictability of 81% to infer the inversion in heterozygosis (as shown in the ROC curve, Figure 5B).

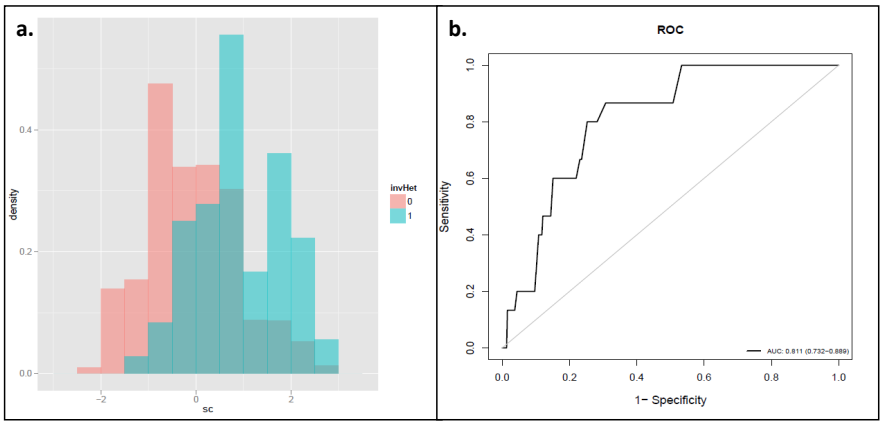

Figure 5. a. Multidimensional approach to discriminate between inversion and no-inversion carriers using the selected SNP genotypes. **b.** Receiver operating characteristic (ROC) curve.

Copy Number Variants are susceptibility alleles for WBS deletion

The frequency of polymorphic blocks CNVs (deletion or duplications) was compared between WBS-transmitting and non-transmitting progenitors. We found an increased frequency of polymorphic block CNVs in the WBS-transmitting group, 4.1% (17/413), compared to non-transmitters, 1.9% (8/413) (χ^2 (1, n=826)=3.34, p=0.067) (Supplementary Table S1). Deletions were 1.7 times more frequent in WBS-transmitters (1.69% vs. 0.97%) and duplications were approximately 2.5 times more frequent (2.42% vs. 0.97%). We observed that 12 of the 17 polymorphic block CNVs were of maternal origin.

DISCUSSION

In this study we have performed a molecular characterization of the *de novo* submicroscopic 7q11.23 deletion in a cohort of 720 unrelated patients, including a detailed analysis of breakpoints in 177 cases. Our studies take advantage of several techniques to define the mechanisms participating in the rearrangements that include microsatellite analyses to determine the deletion extension and identify the SD blocks involved, site-specific nucleotide analysis to delimit the breakpoint, and amplifying and sequencing the junction-fragment. We have successfully defined the size of the deletion, the parental origin and the breakpoint location. We have also identified susceptibility factors that could influence NAHR mechanisms.

Atypical deletions

The frequency of atypical deletions in our cohort was of 1.6%. Nine of them were larger than the typical deletion; while the other three (Antonell et al. 2010a; Delgado et al. 2013), previously described and characterized, were smaller. It can be inferred that other mechanisms different from NAHR could have mediated these deletions since they have occurred outside the typical SDs and are non-recurrent.

The majority of WBS deletions arise due to crossover events between B-blocks

In our unprecedented large cohort of 708 individuals with WBS, we have detected that 86.4% cases occur due to crossing-over events between the Bc and Bm blocks generating a 1.55 Mb deletion which is in agreement with our previous publication (Bayés et al. 2003). The remaining 12% of patients have a larger deletion size of 1.83 Mb, secondary to rearrangements between the Ac and Am blocks, also in concordance with previous results. The recombination rate observed in WBS is in agreement with what has been previously stated (Turner et al. 2008), the recombination rate is increased in the B blocks because of a higher degree of sequence homology between the blocks Bc and Bm (99.6%) compared to the Ac and Am blocks (98.2%) and the shorter distance between both duplicons (Bayés et al. 2003).

Contrary to an increased female recombination rate observed for deletions at 22q11.2 (Delio et al. 2013), we did not find significant differences in parental origin of the deletions.

The majority of 1.55 Mb deletions tend to cluster in the proximal half of the block Bc/Bm containing

GTF2I/GTF2IP1 and *NCF1/NCF1P1* genes

We have refined the location of the breakpoint 1.55 Mb deletions by the analysis of five of the thirteen originally defined site-specific nucleotides in 177 samples (Bayés et al. 2003). As it has been previously described, PCR amplification followed by restriction enzyme analysis allows the relative quantification of the medial, centromeric and telomeric B blocks to determine where the breakpoint has occurred (Bayés et al. 2003). In agreement with our previous results (Bayés et al. 2003), we corroborate in this larger cohort that, although crossing over events can occur at any position, approximately 76% of the deletions occur at the proximal half of the block that contains *GTF2I/GTF2IP1* and *NCF1/NCF1P1* genes. Half of these deletions occur before *NCF1* gene. In the cases that we have been able to refine the breakpoint location, 70% are located between exon 16 and 21 of *GTF2I*. There are various factors that could explain the observed preferential location, the first half of Bc and Bm has a higher percentage of identity (99.8%) compared to the second half (99.4%) and it also contains five of the seven stretches of continuous identity (Bayés et al. 2003). This corroborates our initial hypothesis that crossing-over events that generate the 1.55 Mb deletion occur in regions of very high sequence identity (Bayés et al. 2003).

Deletions located within the *NCF1* gene were less frequent (16.2%). The breakpoint located at *GTF2IRD2* occurred in approximately 27% of cases, generating a chimeric copy, with the first exons corresponding to the medial B block and the final exons to the centromeric copy. The functional implications of this *GTF2IRD2* molecular variant are still unknown.

We also observed a paternal sex-bias with the deletions occurring before *NCF1* gene, result that is maintained when the cohort is increased by five times (Bayés et al. 2003).

The presence of a paracentric inversion predisposes WBS deletion in 22% of cases

The presence of SDs in inverse orientation can predispose to the occurrence of inversions (Turner et al. 2008), as is the case of the paracentric inversion at 7q11.23. The exact size of the inversion is unknown since the crossing-over can occur between any centromeric and telomeric blocks (Bayés et al. 2003). During meiosis I the inverted and the normal chromosome form a structural micro-loop in the middle of the inverted segment (Bayés et al. 2003). The crossover between an

inverted and a non-inverted chromosome occurs at the last 38kb absent in the centromeric B block, since this region of Bm and Bt are most likely to be misaligned (Bayés et al. 2003). The crossing over should occur between the Bt and Bm to result in a deletion or duplication, crossing over at other regions can result in an acrocentric or dicentric chromosome that wont segregate correctly during meiosis (Bayés et al. 2003). SSN11 analysis allows us to infer whether the deletion was mediated by an inversion, since we can detect a gain of the Bt block. Corroborating previous findings, the deletion was mediated by a paracentric inversion in the transmitting progenitor in approximately 22% of our patients (Bayés et al. 2003; Hobart et al. 2010; Osborne et al. 2001). The presence of the inversion in progenitors of WBS, inferred by SSN11 assay, was confirmed by FISH analysis in all the individuals studied (7/7) and no parental origin bias was observed.

The presence of the paracentric inversion can predispose to the occurrence of chromosomal rearrangements secondary to chromosomal mispairing during meiosis. This has also been observed in other recurrent genomic disorders, for example Sotos syndrome or Angelman syndrome, in which the frequency of inversions in the transmitting progenitors was increased compared to the frequency of the inversion in the general population (Visser et al. 2005; Gimelli et al. 2003). In certain disorders, like the microdeletion at 17q21.31, the presence of an inversion is a requisite for the rearrangement to occur, since all the transmitting progenitors are carriers of the inversion (Shaw-Smith et al. 2006; Koolen et al. 2008).

We analyzed a novel SSN to refine the breakpoint location (SSN11A) and we found that in 71% of cases the breakpoint was located distally from the intron 2 of *GTF2IRD2*.

Positional preference for strand exchange in 7q11.23 deletions

In many recurrent genomic disorders a positional preference for strand exchange has been identified. For instance, a 12 kb hotspot was described for Smith-Magenis syndrome (SMS, OMIM 182290), a 3.4 kb hotspot was present in 46% of Neurofibromatosis type I deletions (NF1, OMIM 162200) (López-Correa et al. 2001; Bi et al. 2003) and a 1.7 kb hotspot has been described in the deletion and reciprocal duplication that cause the hereditary neuropathy with liability to pressure palsies (HNPP, OMIM 162500) and Charcot-Marie-Tooth type 1A (CMT1A, OMIM 118220)(Reiter et al. 1996a).

We have previously described a 3.4 kb hotspot within *GTF2I* for the deletions of 1.55 Mb with a crossover between the Bc and Bm blocks (Bayés et al. 2003) that we corroborate in this study. We have studied 73 cases with deletions located at the proximal half of the block, of which 33% amplified *de novo* 3.4 kb junction fragment. We can infer the expected number of *de novo* JF-GTF2I by multiplying this percentage (33%) by the total number of deletions with a putative breakpoint at this region (n=83), obtaining approximately 27 *de novo* JF-GTF2I. Therefore, the rate of recombination at this hotspot is 7.5 events per kb. This is much higher than the global recombination rate at WBS critical region, which is approximately 1.6 events per kb.

In agreement with previous data, this hotspot occurs almost exclusively in deletions of paternal origin (17 compared to 1). In 10 families, for which we had sibling's samples available for haplotype analysis of 7q11.23, we studied whether the deletion arose by an intrachromosomal or an interchromosomic rearrangement (from previous and novel data (Bayés et al. 2003)). In 7 the deletions arose through an intrachromosomal event. Therefore, it seems that rearrangements at JF-GTF2I hotspot occurs almost exclusively by an intrachromosomal event in paternal meiosis.

Sex-preferential hotspots have also been observed in other genomic disorders. The majority of CMT1A duplications and 85% of spinal muscle atrophy (SMA, OMIM 253300) deletions had a paternal origin (Wirth et al. 1997; Lopes et al. 1997). Whereas, 80% of NF1 deletions are of maternal origin (López-Correa et al. 2001).

In the case of the inversion-mediated deletions, we describe two novel hotspots located within *GTF2IRD2*, JFINV1 of 1.4 kb and JFINV2 of 2.6 kb. Of the 59 inversion-mediated deletions, 9 amplified a *de novo* JFINV1 (15.8%) and 22 amplified JFINV2 (38.6%). The rate of recombination for JFINV1 is 6.4 events per kb and for JFINV2 is 8.5 events per kb. Also, much higher compared to the global recombination rate for inversion-mediated deletions (approximately 1.6 events per kb).

Although there was no significant sex-bias observed in these hotspots, there was a slight increased frequency of paternal deletions in JFVIN1 and of maternal deletions in JFINV2.

It might seem that different sex-dependent factors act in distinct recombination mechanisms, determining the preference for crossovers. It is known that the distribution of crossover locations is

different between both sexes, females tend to have lower frequency at the telomeres and higher near the centromeres compared to males (Broman et al. 1998).

Cis and *trans*-acting mechanisms involved in NAHR

The *GTF2I* hotspot accounts for only 1.5% of the 105 kb of Bc/Bm non-allelic pairing, while the other two hotspots within *GTF2IRD2* account for 10% of the 38 kb Bt/Bm non-allelic pairing. In order to explain the positional preference for recombination within these short intervals, we scrutinized all three hotspots and their 5 kb flanking regions for evidence of strand exchange promoting sequences and structures. We calculated the percentage of GC content and observed a uniform distribution along the entire B block. However, there was a higher density of repetitive elements in the last 38 kb of the B block that aligns during meiosis in heterozygous inversion carriers. The three hotspots contained a significant higher density of *Alu* repeats compared to the rest of block B. We also detected an increase of the immunoglobin heavy class switch repeats in all three hotspots, compared to the distribution in the B block and specific recombination motifs at each of the junction fragments (DNA polymerase α/β frameshift motif in JF-GTF2I and the ARS consensus from *S. pombe* in JFINV1). The enrichment of recombination motifs has also been described in other hotspots of recurrent genomic disorders (Visser et al. 2005; Shaw et al. 2004; Bi et al. 2003; López-Correa et al. 2001; Reiter et al. 1996b). A *mariner*-like element (MITE) was identified in the 1.7 kb hotspot at 17p11.2 described in Charcot-Marie-Tooth type IA (CMTIA, OMIM 118220) and Hereditary Neuropathy with liability to pressure palsies (HNPP, OMIM 162500)(Reiter et al. 1996b). In the case of Sotos syndrome (OMIM 614753), caused by a deletion at 5q35, the 3kb-hotspot described also contained various recombination motifs, including translin target sites and scaffold attachment regions (Visser et al. 2005). The majority of the junctions of the 524 bp hotspot described for uncommon deletions of SMS, were located within an *Alu* element (Shaw et al. 2004). *Alu* sequences can trigger NAHR events leading to deletions, duplications and other chromosomal abnormalities (Batzer and Deininger 2002; Deininger and Batzer 1999; Sen et al. 2006). It was reported that NAHR between *Alu* repeats were involved in 33 germ-line diseases and in 16 cases of cancer (Deininger and Batzer 1999). It has also been

reported that the presence of a fixed *Alu* repeat can increase the recombination rate by approximately 6% (Witherspoon et al. 2009). The *Alu* repeats present in the three WBS hotspots might explain why there is an increased recombination rate at these regions.

Trans-acting mechanisms can also influence the rate of recombination. A 13-mer motif was identified to be involved in approximately 40% of human hotspots (Myers et al. 2008). It was later found that this motif was the binding site of a highly polymorphic zinc finger protein, *PRDM9* (Berg et al. 2010). This protein is highly expressed during the early stages of meiosis in germ cells and specifies hotspot usage by binding to the motif (Hayashi et al. 2005). Crossover frequencies in sperm donors showed a significant lower recombination among individuals with non-A alleles (Berg et al. 2010). The authors also studied the rate of *de novo* rearrangements at 17p11.2 associated with NHPP and CMT1A and found that non-A alleles were protective against recombination at this locus (Berg et al. 2010). Other studies have also been done in other genomic disorders with conflicting results. A trio study of progenitors of WBS patients found an increased frequency of non-A alleles in the transmitting group compared to the non-transmitting. However, the great majority of transmitting progenitors (≈91%) were homozygous for the A-allele (Borel et al. 2012). Another study using the same strategy found an increased frequency of the A-allele in transmitting progenitors of a *de novo* 22q11.2 deletion (Alemany-Schmidt et al. 2014). In both studies the cohorts studied were fairly small, 17 and 16 trios, respectively (Borel et al. 2012; Alemany-Schmidt et al. 2014). In our study, we did not find any statistical difference between the transmitting and non-transmitting groups. The allelic frequencies described in our cohort (A-allele 0.921 and non-A alleles 0.079) are similar to what has been described in other studies (Borel et al. 2012). Even though we did not find any relationship with PRDM9 genotype, we did find a slight increase in the frequency of the 13-mer motif recognized by *PRDM9* in two of the hotspots, JF-GTF2I and JFINV2, compared to the rest of the B block. It seems that the effect of *PRDM9* on the recombination rate might be specific among genomic disorders.

Paracentric inversion at 7q11.23

We studied the presence of the inversion by FISH analysis in 48 controls, and found a frequency of 4.2%, in agreement with previous data (Bayés et al. 2003; Hobart et al. 2010; Osborne et al. 2001). In order to investigate whether most

inversions had originated from a common ancestor or through recurrent genomic mutations, we determined the haplotypes associated with the inversion polymorphism using siblings and/or grandparents to establish the phase. We did not find a single haplotype shared by the eight chromosomes identified with the inversion. The inversion events probably occur as independent mutational events in multiple founders.

Inversions are very difficult to study using experimental techniques. Recently, two bio-informatic approaches have been created to study inversions in a genome-wide level based on the fact that chromosomal inversions are known to suppress local recombination in heterozygous carriers and, therefore, the presence of inversions may be associated with blocks of linkage disequilibrium (LD) (Cáceres et al. 2012; Cáceres and González 2015). InveRsion is an algorithm that detects changes of LD at the breakpoints of the deletion by studying ≈10 SNPs flanking the breakpoint in 0.4 Mb windows (Cáceres et al. 2012). InvClust studies all the SNPs inside the complete inverted segment searching for the existence of extended haplotypes (Cáceres and González 2015). Both approaches work best for ancestral inversions with no or very low recurrence (Cáceres et al. 2012; Cáceres and González 2015). We used both approaches to detect tag SNPs that could infer the paracentric inversion at 7q11.23. We chose five SNPs and genotyped them in transmitting progenitors that were heterozygous carriers for the inversion and also in WBS individuals, hemizygous for the deletion. We found a statistically significant difference of the allelic and genotypic frequencies of three SNPs between the inversion-carrier group and the no-inversion group. We also found five haplotypes that were at least three times more frequent in inversion carriers compared to non-inversion carriers. Using a multidimensional method or a prediction method based on ORs, we have a predictability of 63% and 81% to infer an inversion in heterozygosis, respectively. This approach has also been used to detect inversions based on SNP array data (González et al. 2014).

Other copy number variants at 7q11.23

Due to the complex genomic architecture of the WBS critical region, other structural variants, apart from the paracentric inversion, can occur. We reported previously the presence of deletions and duplications of the SDs flanking the region that were in enriched in progenitors of WBS patients, compared to the general population

(Cuscó et al. 2008). We corroborate this previous finding in a larger cohort of transmitting progenitors; we found a 2.8 fold increase of structural variants in the WBS transmitting group compared to the non-transmitting group. Although we did not find any significant association with the parental origin of the deletion, the majority of variants were of maternal origin. Similarly to the paracentric inversion, structural variants of the SDs flanking the region can predispose to chromosomal misalignment during meiosis making them susceptible to rearrangements (Bayés et al. 2003; Cuscó et al. 2008).

Gene-conversion events at 7q11.23

Gene conversion is thought to be mechanistically related with homologous recombination, given that the resolution of double Holliday junctions could lead to homologous recombination and/or gene conversion events (Jeffreys and May 2004). Our data based on SSN genotyping and sequence analysis across a large number of independent recombination events at the three WBS-hotspots, fully support this hypothesis (Table S2). We observed contiguous stretches of *trans*-morphisms surrounding or overlapping the hotspot intervals in WBS patients, and they could also be inferred in chromosomes from transmitting and non-transmitting progenitors, suggesting gene conversion events likely associated with heteroduplex repair. For instance, gene conversion events at *NCF1* gene are present in approximately 11.3% of WBS progenitors.

In summary we have corroborated our previous findings in a larger cohort of patients with WBS and contributed with novel findings regarding the mechanisms involved. We corroborate in a larger cohort the presence of a 3.4 kb hotspot within *GTF2I* gene present almost exclusively in paternal meiosis and describe two novel hotspots within *GTF2IRD2* for the inversion-mediated deletions. It seems that within WBS deletion there are sex-dependent factors that increase the recombination rate at specific regions. It would be interesting to determine which protein or proteins play a role in generating an intrachromosomal rearrangement at JF-GTF2I in male meiosis.

The aim of our studies is to increase the knowledge of the mechanisms involved in NAHR, elucidating on the putative *cis* and *trans*-acting mechanisms that can influence the rate of recombination. A detailed molecular characterization of the deletion also allows researches to carry on phenotype-genotype correlations to gain insight for the genes involved in the deletion.

Further studies are needed to corroborate possible sex-dependent factors involved in meiotic recombination.

MATERIAL AND METHODS

Subjects

We have studied a total of 720 individuals with *de novo* submicroscopic deletions at the WBS locus on chromosomal band 7q11.23. Patients were referred on the basis of a clinical suspicion of WBS or after the documentation of a 7q11.23 heterozygous deletion by FISH analysis. Individuals were referred from Spanish centers (n=281), other European (n=42) and North (n=359) and South American centers (n=38). The study was approved by the Institutional Review Board and Ethics Committee and informed consent was obtained from parents or caregivers. Samples from both parents were obtained for all available cases (n=402) and from unaffected siblings in 40 families. Genomic DNA, as well as chromosome slides in some cases, was obtained from peripheral blood, in accordance with standard methods. Total RNA of selected patients with specific deletions (n=30) was also obtained from peripheral blood cells following the trizol protocol.

Molecular characterization of the rearrangements at 7q11.23

The size and parental origin of the rearrangements at 7q11.23 were established by a battery of assays; including PCR analysis of single and multiple-copy microsatellite, as described in detail elsewhere (Bayés et al. 2003).

Site Specific Nucleotides (SSN) Assays

Dosage analysis of site specific nucleotides (SSNs) or paralogous sequence variants (PSV) has been successfully used to refine the breakpoints of the common WBS deletions within the repetitive block B sequence (Bayés et al. 2003). In general, most SSNs correspond to single-nucleotide changes that were detected by the use of restriction enzymes followed by size fractionation on 1%–3% agarose gels. Primer sequence and PCR conditions are described in detail elsewhere (Bayés et al. 2003).

Specific SSNs from the original 13 previously described, were genotyped to identify the site of strand exchange due to their lower polymorphism rate and informativeness (Figure 1). These include SSN2 located at exon 21 of the general transcription factor II-I (*GTF2I* OMIM 601679), SSN4 to estimate the copy number of neutrophil cytosolic factor 1 gene (*NCF1*, OMIM 608512) (block Bm)

and pseudogenes (blocks Bc and Bt), SSN 7 and SSN 9 to infer the molecular variants of GTF2I repeat domain-containing protein 2 (*GTF2IRD2*, OMIM 608899) and SSN 11 (within *GTF2IRD2*, close to the end of the ~105-kb alignment between Bc and Bm/Bt) to determine the frequency of inversion-mediated deletions in patients with 1.55 Mb deletions.

To more precisely refine the sites of crossover in our series of WBS patients with inversion-mediated deletion, an additional SSN has been genotyped (SSNs 11a). PCR primers, their position and the detection procedure are listed in Supplementary Table 2. The degree of polymorphic variation (*trans*-morphisms) of the novel SSN was calculated in the progenitors of WBS patients (Table S2).

As previously described (Bayés et al. 2003), to estimate the relative number of Block B copies, a digital image of the gel is captured at varying exposure times to ensure that the bands are not saturated. Then, intensities of bands corresponding to the presumed blocks Bc, Bm and/or Bt are quantified using the Volume Tool from the Quantity One Software package (Bio-Rad). Relative intensities were calculated by means of a dosage quotient for the block that can be distinguished in a particular SSN assay relative to the other amplified blocks. This block B dosage quotient was calculated for all patients, as well as for their parents. A final ratio "called patient/progenitor block B ratio," was calculated by relating the dosage obtained for each individual to the mean dosage value of the control individuals from the same gel. The relative quantification of SSNs among the centromeric (Bc), medial (Bm) and telomeric (Bt) blocks B in patients with respect to their parents allowed us to define the number of block-B type copies at each position analyzed and then to infer the copy type at the recombinant block B in the patient. By testing specific SSNs, we identified the site of transition from Bc (or Bt in inversion carriers) to Bm in each WBS patient.

Detection of de novo junction fragments in 7q11.23 deletions

We previously reported a 3.4 kb recurrent deletion junction fragment within the *GTF2I* gene (JF-GTF2I-hotspot) in WBS patients with the 1.55 Mb deletion (Bayés et al. 2003). To screen this NAHR hotspot, we specifically amplified the putative 3.4 kb *de novo* deletion-junction fragment using site-specific and internal control primers as described elsewhere (Bayés et al. 2003). This assay was performed in 73 WBS patients (out of 83) in whom the breakpoint was suggested to be located in that 3.4-kb interval,

centromeric to the SSN2. In 18 cases we were not able to amplify the junction fragment due to low integrity DNA.

We designed additional PCR-based *de novo* fragment detection for inversion-mediated deletions. To amplify the most proximal 1.4 kb de novo junction fragment (JFINV1) we designed a nested PCR. The primers used for the first reaction were GTF2IBF and BSTNR, as shown in Table 2, with an amplicon size of 1.5 kb. For the second reaction, the forward primer was designed with two mismatches (shown in boldface in Supplementary Table 3) specific for the telomeric block (Tru9Tel) and the reverse primer was complementary to both medial and telomeric B-blocks (BstnMED). As an internal positive control, we designed a second forward primer specific for the medial block (True9Med) that amplifies a 1.4 kb fragment.

To amplify the most distal 2.6 kb de novo junction fragment (JFINV2), we designed a forward primer that binds to both medial and telomeric B-blocks (FusioF) and a specific reverse primer for the telomeric B-block (FusioR1), as shown in Table S3. An internal positive control PCR was designed using the same forward primer with a specific reverse primer for the medial B-block (FusR2).

If a deletion-junction fragment was also amplified in the progenitor's samples, we purified from agarose gels (QIAGEN) and sequenced the products to assess if the amplification was secondary to an inherited polymorphism. Internal primers were designed for each JFINV for Sanger sequencing. These assays were performed in 57 of the 59 patients with the inversion-mediated deletion (97%). In 2 samples we were unable to amplify the junction fragment due to low integrity DNA.

Expression Analysis of *GTF2IRD2* Transcripts

RNA was extracted from Epstein-Barr virus transformed LCLs (Antonell et al. 2010b) from 13 individuals with WBS with different molecular variants of *GTF2IRD2* and from 4 control individuals each cell line using TRIZOL reagent (Invitrogen) according to manufacturer's instructions. cDNA was prepared from 1 μg total RNA using random hexamers and SuperScript II RNase reverse transcriptase (Invitrogen).

PCR amplification from cDNA was done using a primer at exon 11 and the reverse at exon 16 (Table S4) of *GTF2IRD2* followed by band purification using QIAQuick Gel Extraction Kit (QIAGEN) according to manufacturers instructions. *GTF2IRD2* was

Sanger-sequenced to analyze PSVs and determine whether the chimera molecular variant was expressed.

A second PCR-amplification reaction was performed from cDNA using the same forward primer and a reverse located at the end of exon 16 (Table S4), followed by band purification using illustra GFX PCR, DNA and Gel Band Purification kit (GE Healthcare) according to manufacturers instructions. The PCR product was directly introduced into the pCR 2.1 TOPO vector (Invitrogen) using TA cloning. The subclones were Sanger-sequenced.

Fluorescent In Situ Hybridization (FISH) Analysis

Chromosome spreads for interphase FISH analysis were prepared from peripheral blood lymphocytes using standard methodology. Three-color FISH was performed as described elsewhere (Bayés et al. 2003). FISH was performed in seven inversion carriers previously predicted by SSN-typing. To establish the prevalence of the inversion polymorphism in the general population, three-color FISH in interphase nuclei was done in a cohort of 48 controls, including 25 non-transmitting WBS parents.

Sequence analysis

Sequence motifs associated with recombination and replication events (plus their complements) were searched at all three hotspots and their 5 kb flanking regions as described elsewhere (Badge et al. 2000; Abeysinghe et al. 2003). RepeatMasker was run to identify interspersed repeats and the GC content in the region. Finally, using BLAST algorithms we compared the sequence of the three hotspots with each other and with all other reported NAHR hotspots associated with genomic disorders.

***PRDM9* Genotype**

PR domain containing protein 9 (*PRDM9,* OMIM 609760) alleles were genotyped in a cohort of 161 WBS-transmitting progenitors and 168 non-transmitting individuals (144 non-transmitting progenitors and 24 controls). PCR amplification was performed with the primers PN0.6F (5´-TGAGGTTACCTAGTCTGGCA-3´) and PN2.5R (5´-ATAAGGGGTCAGCAGACTTC-3´), followed by an internal PCR using PN1.2F (5´-TGAATCCAGGGAACACAGGC-3´) and PN2.4R (5´-GCAAGTGTGTGGKGACCACA-3´) (Berg et al. 2010). PCR product was digested with *Bst*UI (New England Biolabs) enzyme to discriminate A and B-alleles from the majority of non-A alleles. We

were able to discriminate based on the amplicon size and if it was digested by the enzyme. We designated each of the different observed alleles based on the reported allelic frequency for European population. We compared the frequency of *PRDM9* alleles between the WBS deletion transmitters to non-transmitters.

Haplotype Analysis in Inversion carriers

Haplotypes associated with the inversion polymorphism were determined by genotyping 11 microsatellites and PCR-RFLPs distributed along the 7q11.23 region (from D7S672 to D7D2158, including 7 markers within the common WBS deleted interval), using siblings and/or grandparents to establish the phase (n=8).

Single nucleotide polymorphisms (SNPs) in 7q11.23 were analyzed by the bioinformatic tools InveRsion and invClust (Cáceres et al. 2012; Cáceres and González 2015). Five SNPs predicted to infer the inversion were selected (rs1109512, rs2528997, rs4717820, rs6460068) and rs799160 also located in WBS region. These six SNPs were genotyped in a cohort of 275 WBS patients and in 71 progenitors-carriers of the 7q11.23 paracentric inversion. The SNPs were genotyped using the Sequenom MassArray iPLEX system (Sequenom Inc.). Two HapMap samples and a trio were included in the assay for quality control, and no discordant genotypes were found.

Statistical analyses

Statistical analyses were performed using the package SPSS 19.0 according to the characteristics of each variable. Specifically Chi-squared and Fisher-test were used when needed. A p-value <0.05 denoted the presence of statistically significant differences.

For SNP association study, Hardy-Weinberg equilibrium and haplotype construction were done using SNPstats (Solé et al. 2006). Only rs799160 was deviated from equilibrium in the inversion-carrier group ($p=0.0026$). The allelic and haplotype frequency were compared between inversion and non-inversion carriers groups. We constructed the homozygous individuals haplotypes from the hemizygous haplotypes of non-inversion carriers (WBS patients), and we also compared the allelic and haplotypic frequency between both groups. The haplotype predictability was assessed by a multidimensional approach and a prediction based on Odds Ratio.

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DISCLOSURE DECLARATION

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ELECTRONIC DATABASE INFORMATION

URLs for data presented herein are as follows:

RepeatMasker: http://woody.embl-heidelberg.de/repeatmask/

Primer3: http://www-genome.wi.mit.edu/genome-software/other/primer3.html

Online Mendelian Inheritance in Man (OMIM): for WBS, CMT1A, HNPP, NF1, SMS, SMA and SoS.

http://www.ncbi.nlm.nih.gov/Omim/

BLAST2 Sequences: for pairwise sequence comparison.

http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi

BioEdit software:

http://www.mbio.ncsu.edu/RNaseP/info/programs/BIOEDIT/bioedit.html

EMBOSS GUI: for sequence analysis

http://bioinfo2.ugr.es/EMBOSS-GUI/

Statistics: for Fisher's exact and $\chi 2$ tests

http://www.unc.edu/~preacher/

SNPstats

http://bioinfo.iconcologia.net/SNPstats

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SUPPLEMENTARY TABLES

Table S1. Polymorphic block CNVs in transmitters and non-transmitter progenitors.

Polymorphic block CNV	WBS-transmitter progenitors	Non-transmitter progenitors	Odds Ratio (CI95)	p-value
Deletion	7/413	4/413	1.762 (0.512-6.07)	0.369
Duplication	10/413	4/413	2.54 (0.789-8.16)	0.176
Total	17/413	8/413	2.17 (0.927-5.09)	0.067

Table S2. SSNs used for mapping the deletion breakpoint in patients with 1.55 Mb deletion.

SSN	Position[1]	Primers	Detection procedure	
2	16,572 bp	F 5´-CTCAAGCTCTTGGACTCAC-3´	*Bst*UI	Bc and Bt: 397, 759
	GTF2I (exon 21)	R 5´-ATCCCAGGAGGCAAGTAGGAAAT-3´		Bm: 151, 246, 759
4	48,967 bp	F 5´-TCCCCCGACTCTGGCTTTC-3´	delGT	Bc and Bt: 103
	NCF1 (exon 2)	R 5´-GGGGAGCTTGAGGTCATCAG-3´		Bm: 105
7	68,364 bp	F 5´-ATGAATAGTGAGGCATACAATG-3´	*Bst*UI	Bc: 499, 222, 147, 243
	GTF2IRD2 (exon 16)	R 5´-CCATAGATTGGATCCGAGACCT-3´		Bm and Bt: 868, 243
11	104,958 bp	F 5´-TTGTAAAATGGTGTTTATTTTAGG-3´	*Tru9*I	Bt: 20, 242, 51, 156
	GTF2IRD2 (intron 3)	R 5´- GCCCCACAAACTTGGATCTG-3´		Bc and Bm: 20, 100, 142, 51, 156
11a	106,305 bp	F 5'-TTAGAATGAGAAAAATCACTGAGAAA-3'	*Bst*N	Bt: 350,300
	GTF2IRD2 (intron 2)	R 5'-ATAATAAAGCATTAGGATCTGTTTGA-3'		Bm: 350,255,45

[1] Nucleotide numbers correspond to the Bm consensus sequence. [2] The degree of polymorphism for SSN4, 2 and 11a were calculated by genotyping progenitors of WBS patients. The number of chromosomes with abnormal block B dosages is shown. [3] Data obtained from (Bayés et al. 2003).

Table S3. Site specific PCR primers to amplify across Bt/Bm inter-chromosomal junction fragments (site-specific mismatches are in boldface).

Primer name	Sequence (5'-3')	Tm (°C)	Combination	PCR product
Gtf2iBF	TTGTAAAATGGTGTTTATTTTAGGAA	57.5 °C		
BstnR	TCAAACAGATCCTAATGCTTTATTAT	56.9 °C	Gtf2ibF + BstnR	JFINV1 first reaction
Tru9Tel	GACTCTGCAGTGTTAGGCAGTA	57.3 °C	Tru9Tel + BstnMed	Proximal 1.4 kb junction fragment (JFINV1)
True9Med	GACTCTGCAGTGTTAAGCAGTG	57.8 °C	True9Med + BstnMed	1.4 kb internal control
BstnMed	CTCGAAATATCTTCCCCAGG	58.6 °C		
FusioF	ACTATTTCACTTACACATAGAAAGGA	54.9 °C		
FusioR1	CATAGATCAATGGCAATTGG	56.5 °C	FusioF + FusioR1	Distal 2,6 kb junction fragment
FusR2	GCATCCTACTGGTTCAGCA	57.3 °C	FusioF + FusR2	1.5 kb internal control
JFINV1A	ACGGAGTCTCGCTCTGTAC	64.8 °C		Sanger sequencing JFINV1
JFINV1B	CTGCCTGACATGGATCTTCC	64.7 °C		Sanger sequencing JFINV1
JFINV2A	ATGCGGAAGGAAGACAGAAG	63.3 °C		Sanger sequencing JFINV2
JFINV2B	CCACCACACCTGGCTAACTT	63.9 °C		Sanger sequencing JFINV2

Table S4. PCR primers used to amplify and Sanger-sequence *GTF2IRD2* paralogous sequence variants (PSVs).

Primer Name	Exon	Sequence (5´-3´)	Tm (C°)	PCR Product
GTF2IL-E11F	11	5´-CGAGGTGAAAATCGAAGGAA-3´	60.2 °C	-
GTF2IL-E15R	16	5´-ATCGACACCACGGATGAATATGG-3´	65.1 °C	859 bp
GTF2IRD2-E15R3	16	5´-AATCCCAGCACTTTGTGAGGTCG-3´	66.7 °C	2350 bp

CHAPTER 2

Metabolic abnormalities in Williams-Beuren syndrome

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Reported metabolic alterations in WBS include transient hypercalcemia of infancy, subclinical hypothyrodisim in approximately 30% of children and glucose metabolism alterations in up to 75% of adults with WBS.

In this study we have analyzed several metabolic parameters in a cohort of 154 WBS patients (data available from 69 to 151 cases per parameter), as well as several mouse models with complete and partial deletions of the orthologous WBS locus and searched for causative genes and potential modifiers.

Triglyceride plasma levels were decreased in patients with WBS, with cholesterol levels slightly decreased compared to controls. Approximately 18% of patients presented hyperbilirubinemia, mostly unconjugated, and correlated with subclinical hypothyroidism and hypotriglyceridemia, suggesting common pathogenic mechanisms. Haploinsufficiency at *MLXIPL* and increased penetrance of the hypomorphic allele at the *UGT1A1* gene promoter might be associated with the lipid and bilirubin alterations. Other unreported frequent metabolic alterations were increased total protein and albumin levels, as well as alterations in iron metabolism. Known disturbances, such as subclinical hypothyroidism and glucose intolerance, were also described.

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CHAPTER 3

Cardiovascular manifestations and evaluation in Williams-Beuren syndrome

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In preparation

Cardiovascular manifestations occur in approximately 75% of WBS patients and are characterized by stenosis of medium and large-sized arteries; the most frequent locations are SVAS and PPS. HTN prevalence in WBS is variable, although approximately 50% of patients develop it. Few studies have been done studying ambulatory blood pressure in patients with WBS.

In this study we have analyzed clinical information about cardiovascular disease and hypertension in 136 patients with WBS. In a smaller cohort of patients we have analyzed in detail ambulatory blood pressure monitoring and included echocardiographic, abdominal Doppler ultrasound and, in few cases, CT angiogram evaluation. We treated with losartan hypertensive patients with a follow-up visit after 12-months. We have also studied of renin-angiotensin-aldosterone axis and markers of oxidative stress damage. Finally, an association study has also been performed to identify possible genetic modifiers of the cardiovascular phenotype.

We have identified an important number of patients that present a systemic arteriopathy with various degrees of affection. CT angiogram seems to be a better diagnostic technique than abdominal Doppler ultrasound for its diagnosis. We also report an important number of patients with nocturnal hypertension and/or blunted nocturnal decrease. LVH and altered LV geometry are significantly associated with hypertension. We also describe novel genetic variants associated with cardiovascular disease and arterial hypertension.

Cardiovascular manifestations and evaluation in Williams-Beuren Syndrome

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ABSTRACT

Williams-Beuren syndrome (WBS, OMIM 194050) is a neurocognitive disorder with multisystemic manifestations of variable expressivity caused by 1.55–1.83 Mb hemizygous deletion at chromosome band 7q11.23. One of the main clinical manifestations in WBS is cardiovascular disease present in approximately 75% of patients. The hallmark of cardiovascular disease in WBS patients is the stenosis of medium and large sized arteries. Another frequent cardiovascular manifestation is arterial hypertension present in approximately 50% of patients. Few studies have been done using ambulatory blood pressure monitoring to determine the prevalence of ambulatory hypertension and its characteristics in WBS. The purpose of the present study is to describe the cardiovascular alterations, office and ambulatory hypertension in a cohort of WBS patients. We have treated a small cohort of hypertensive patients with an angiotensin II receptor blocker and reevaluated at 12-months after treatment. Finally, we have included the analysis of renin-angiotensin-aldosterone axis and oxidative stress, as well as possible genetic modifying factors.

We found that approximately 79% of WBS presented cardiovascular disease with specific alterations in a similar proportion to what has been reported previously. We also report stenosis of abdominal arteries in 43% of patients detected by Doppler-ultrasound of the supraaorctic and abdominal vessels. However, approximately 80% of patients with a normal Doppler-ultrasound presented abdominal stenosis when a CT angiogram was performed. Approximately 33% of patients presented ambulatory hypertension, with a higher frequency of systolic compared to diastolic blood pressure. Surprisingly, 57% of patients presented nocturnal hypertension and isolated in 28.6%. Office and ambulatory hypertension were significantly associated with an increased body mass index. Hypertension was also significantly associated with left ventricular hypertrophy and alterations in left ventricle geometry. Hypertensive patients treated with losartan had a significant decrease of all blood pressure indexes without adverse effects. Office hypertension was associated with *NCF1* copy number when stratified by gender. We also found a significant association of hypertension with *PLCE1* gene and cardiovascular disease with *PLCE1* and *ELN* gene and valvular disease with *FBLN2*.

Our results corroborate previous reports of cardiovascular manifestations in a larger cohort of patients with WBS. We describe in great detail ambulatory blood pressure monitoring in patients with WBS and the main associations found to hypertension, as well as potential genetic modifiers. Further studies are needed to corroborate our results and to study the potential medical implications, as well as possible changes in management or pharmacological treatment.

INTRODUCTION

Williams Beuren syndrome (WBS, OMIM 194050) is a neurodevelopmental disorder with variable expressivity caused by a deletion of 1.55 to 1.83 Mb at chromosome band 7q11.23 [1]. It has an incidence of 1/7,500 to 1/10,000 individuals [2]. WBS is characterized by dysmorphic features, cardiovascular manifestations, endocrine alterations and intellectual deficit with an uneven neurocognitive profile with relative verbal strengths and a significant weakness for visuospatial construction [3].

Cardiovascular manifestations of WBS occur in approximately 75% of individuals, and include vascular stenosis and arterial hypertension (HTN). The most frequent stenoses are supravalvular aortic stenosis (SVAS) in 70% [4, 5] and peripheral pulmonary artery stenosis (PPS) in up to 45% of individuals with WBS [5–7], although any artery can be affected.

The prevalence of HTN in WBS varies depending on the method for measurement and the diagnostic criteria that are used. Few studies have been done in WBS patients studying 24-hour ambulatory blood pressure. Most have focused on reporting the prevalence of ambulatory HTN and circadian variation [8–10]. However, a more profound analysis of all the variables of ambulatory blood pressure monitoring (ABPM) has not been done.

The consequences of HTN and cardiovascular disease on left ventricular mass (LVM) and geometry has only been reported in one previous study [11]. The authors observed that left ventricular hypertrophy (LVH) may develop from childhood and that WBS patients can also present differences in left ventricular (LV) remodeling or hypertrophy [11].

The main causative factor of the cardiovascular phenotype is the hemizygous deletion of the elastin gene (*ELN*, OMIM 1301610), which encodes for the elastin protein. The decrease and abnormal production of elastin causes numeral histological changes of the vascular wall, including a decrease in elasticity and smooth muscle cell proliferation leading to hyperplasia of the intima [12, 13]. Potential genetic modifying factors of the cardiovascular phenotype include genetic variants in the remaining copy of the elastin gene, *NCF1* copy number and genetic variation in genes associated with cardiovascular development [14–16]. The deletion of a functional copy of *NCF1* gene has been associated to be a protective factor against HTN by reducing Angiotensin II-mediated oxidative stress [15]. The hypertensive phenotype of mice harboring a partial deletion that included *ELN* gene was pharmacology rescued when treated with an angiotensin II-receptor antagonist (losartan) and a NOX inhibitor (apocynin) and also genetically reverted when crossed with an *Ncf1* mutant [17].

In this study we have compiled and analyzed the clinical information about cardiovascular disease and blood pressure measurements in 136 patients with WBS. We have also analyzed numerous ABPM variables in a smaller cohort of patients (n=47), as well as echocardiography, abdominal Doppler

ultrasound (US) and, in 14 cases, thoraco-abdominal CT angiogram. Hypertensive patients detected with ABPM were treated with losartan and reevaluated after 12-months. Finally we have also included the analysis of the renin-angiotensin system and oxidative stress damage markers, and we have tried to search for possible genetic modifiers of the cardiovascular phenotype (including *NCF1* gene copy number and previously described SNPs associated with arterial hypertension or cardiovascular disease).

MATERIAL AND METHODS

Subjects

Information about cardiovascular alterations was analyzed from a cohort of 136 patients with WBS with well-characterized 7q11.23 deletions. Only patients with typical 7q11.23 deletions were included in the study. In 108 patients (108/136, 79.4%) two or more BP measurements were available. Office HTN was defined in children as BP above the 90th percentile for age and gender using the curves of the Report of the Second Task Force on Blood Pressure Control in Children [18] and in adults with BP above 130/90 as defined by the Seventh Report of the Joint National Committee on prevention, detection, evaluation and treatment of high blood pressure [19], as in our previous study [15].

The majority of patients were referred from Spanish medical centers (129/136, 94.9%%) and also from international medical centers from Portugal (3/136), Brazil (2/136) and Venezuela (2/136). The mean age of the cohort was 14.46±9.44 years (range from 0.4-47 years), 80 males (58.8%) and 56 females (41.2%).

Clinical criteria for inclusion in this study have been approved by the Institutional Review Board and Ethics Committees as described elsewhere [20] and informed consents were signed by the patients and the parents.

Molecular characterization of the deletion

The size and parental origin of the deletion were evaluated using PCR analyses of single and multiple copy microsatellites as described in detail elsewhere [21]. Site-specific nucleotides (SSN) have also been studied to determine the breakpoint location of the deletion as reported elsewhere [21].

NCF1 copy number can be determined by PCR analysis using labeled primers, as previously described [15]. A gene / pseudogene quantification is performed by distinguishing the gene from its pseudogenes by the presence of a 2-bp deletion (GT) at the beginning of exon 2 in the pseudogenes.

Ambulatory Blood Pressure Monitoring

BP monitoring was performed in a cohort of 49 individuals from two medical centers in Spain using the Oscar 2 (Sun Tech Medical Inc., Morrisville, NC USA) device.

We calculated manually the 24-hour mean, systolic and diastolic blood pressure as one third of the sum of twice the daytime (16h) mean value plus the nighttime (8h) mean value. Ambulatory HTN was defined as mean blood pressure (MBP), systolic blood pressure (SBP), diastolic blood pressure (DBP) or nocturnal BP above the 95th

percentile for age and gender using specific curves for adults [22] and children [23]. We decided to include in the analyses patients with seven or more diurnal readings and four or more nocturnal readings.

Several variables were calculated from the ABPM measurements. A blood pressure index (BPI) for the systolic (SBPI), diastolic (DBPI) and nocturnal (NPI) blood pressures was calculated for comparisons since we had patients from both genders with a wide age range [24]. The indexes were calculated by dividing the mean value by the 95th percentile reference value for age and gender. An index above 1 corresponds to a mean blood pressure above the 95th percentile. Blood pressure load (BPL) was calculated as the percentage of readings above the 95th percentile for age and gender. This was done for the systolic and diastolic BP separately both during daytime and nighttime [25].

Circadian variation was determined by the dipping status, which is defined as a reduction of 10% in nocturnal MBP with respect to the diurnal MBP values [26]. Dipping status was classified as inverse or reverse (<0% reduction), non-dippers (0-10% reduction), dippers (10-20% reduction) and extreme dippers (>20% reduction)[27].

BP variability defined as the standard deviation (SD) of the MBP during daytime and nighttime [28], was also analyzed.

Arterial stiffness was indirectly measured using the ambulatory pulse pressure (PP) and the ambulatory arterial stiffness index (AASI). Ambulatory PP was calculated as the difference between the systolic and diastolic blood pressure for 24-hour, daytime and nighttime period [29]. AASI is defined as 1 minus the regression slope of diastolic on systolic blood pressure [30] and was calculated individually.

Biochemical studies- Plasma renin activity, aldosterone and oxidative stress

Plasma renin activity (PRA) (n=47) and aldosterone levels (n=43) were analyzed in the cohort of patients evaluated by ABPM (n=49). PRA and aldosterone were not measured in 2 and 6 patients due to technical problems, respectively.

Oxidative stress was determined by measuring a series of lipid peroxidation and protein oxidation biomarkers. Malondealdehyde (MDA) plasma concentration was determined by its diethylthio-barbituric acid adduct (TBA-MDA) after reverse-phase isocratic HPLC separation of the MDA-TBA complex [31, 32]. Plasma protein carbonyl (PC) group levels were evaluated following the 2,4-dinitrophenylhidrazine assay with slight modifications [33, 34]. Analysis of advanced oxidation protein products (AOPPs) was determined spectrophotometrically following the protocol of Witko-Sarsat [31, 35]. Plasma oxidized low density lipoprotein (OxLDL) was measured by ELISA sandwich as previously described [36]. Enzymatic assays were also done to measure oxidative stress. Glutathione peroxidase (GSH-Px) in whole blood was measured following the protocol of Paglia and Valentine [37] using cumene hydroperoxide as an antioxidant of glutathione [36]. Glutathione reductase (P-GR) in plasma was measured by

following the oxidation of NADPH to NADP* during the reduction of oxidized glutathione [38].

Imaging Study

M-mode, two-dimensional, and Doppler echocardiographic examination was performed in a smaller cohort of patients (n=47) at Hospital Vall d'Hebron in Barcelona, Spain. The presence of cardiovascular abnormalities in any of the suprathoracic extracranial aortic main vessels as well as the abdominal vessels including the main iliac branching was recorded, as well as the severity and if the patients underwent surgical treatment. Outflow tracts stenosis, pressure gradients, width of cardiac ventricular walls, ejection fraction and valvular abnormalities were also evaluated.

The interventricular septum and posterior wall thickness were assessed at the end of the diastole. Left ventricle diameter was measured at the end of the diastole and systole. Left ventricle mass was calculated from the M-mode measurements based on the recommendations for chamber quantifications of the European Society of Cardiology [39]. To reduce variability among individuals and the influence of age and height, a left ventricle mass index was calculated by dividing the left ventricle mass was by the height raised to the 2.7 power [40]. This index is better for studying left ventricular hypertrophy (LVH) in cohorts composed of overweight and obese patients [41]. The relative wall thickness ratio was calculated by adding the measurements at the end of diastole of the posterior wall and the septal thickness, and dividing it by the left ventricle internal dimensions [42]. To define LV geometry, a cut-off value of relative wall thickness was set at 0.41 for all patients since this value has been reported as the upper 95% confidence limit [42] and the 95th percentile value of LV mass / height$^{2.7}$ specific for age and gender obtained from De Simone et al. [40]. Ganau et al. defined four patterns of geometric remodeling in essential hypertensive adult patients. LV geometry defined as normal (relative wall thickness and LVM index below the 95th percentile), concentric remodeling of the left ventricle (relative wall thickness above the 95th percentile and LVM index below the 95th percentile), concentric hypertrophy (relative wall thickness and LVM index above the 95th percentile) and eccentric hypertrophy (relative wall thickness below the 95th percentile, but LVM index above the 95th percentile) [42].

Abdominal Doppler US was also performed in this cohort to exclude the presence of arterial stenosis of the abdominal aorta, celiac trunk, and the superior mesenteric and renal artery.

Patients were asked it they wanted to have an abdominal CT performed to better define some of the anomalies detected by Doppler US and to compare the detection rate of both techniques. Fourteen patients accepted to have an abdominal CT, six of these patients had a MBP above the 95th percentile.

Ambulatory Hypertension - Losartan treatment

Patients with a MBP above the 95th percentile for age and sex were prescribed losartan at a dose of 1

mg/kg/day and asked to be followed-up in a year to evaluate ABPM, oxidative stress and plasma renin activity and aldosterone plasma levels. Eight patients returned to the follow-up visit one year later. However, ABPM was not available for one patient due to recording problems.

Polymorphism genotyping

Nine genetic variants previously associated with HTN or cardiovascular alterations were selected. Two single nucleotide polymorphisms (SNP) in the elastin gene (*ELN*, OMIM 130160), rs2071307 and rs2856728, were genotyped in a cohort of 114 WBS patients by PCR amplification (ELN_I20F: 3'-CGCTCTAGACAAGGCCTGGGGGAAATTTACATCC-5' and ELN_I20R: 3'-CGCAAGCTTCTGGAGGCCTGGGAGCCAGTTTG-5') followed by digestion by a restriction enzyme (BstNI). The remaining seven SNPs (Table 1) were genotyped using the Sequenom MassArray iPLEX system (Sequenom) in 122 patients.

Gene	SNP	Evidence
ELN	rs2071307 rs2856728	Encodes a protein that is one of the two components of elastic fibers and is the main causative factor of the cardiovascular pathology in WBS.
ACE	rs4305	Encodes for the angiotensin converting enzyme and has been associated in GWAS studies with HTN, increased SBP and DBP [43].
REN	rs6693954	Alteration in renin activity have been associated with HTN [44].
AGT	rs11122587 rs7079	Angiotensin has been associated with HTN, DBP levels [43, 45].
RYR2	rs2820037	Ryanodine receptor 2 has been associated with high blood pressure and obesity (weight sensitivity increases in SBP and DBP) [46–48].
FBLN2	rs3732666	Encodes an extracellular matrix protein that is involved in configuring, maintaining and integrating the extracellular matrix and basal membrane [49].
CACNB2	rs11014166	Encodes a subunit of a calcium channel protein that has been observed to effect BP regulation and is a target of pharmacological treatment [50].
ZNF5652	rs16948048	5th strongest signal for DBP in GWAS study: 0.34 mmHg increase in DBP per minor allele [51].
PLCE1	rs932764	Phospholipase C plays an important role in podocyte development in the glomerulus [52].

Table 1. Genotyped SNPs associated with hypertension and cardiovascular risk.

Two HapMap samples and a trio were included in the assay for quality control, and no discordant genotypes were found.

Statistical Analysis

All statistical analyses were done with the statistical package SPSS version 19.0 according to the characteristics of each variable. Due to the small sample number in each group, non-parametric tests were used to correlate with categorical variables (U-Mann-Whitney and Wilcoxon test). Pearson correlation was also used to compare numerical variables. A p-value <0.05 denoted the presence of statistically significant differences.

RESULTS

Study population

A total of 136 patients were included in the study with a mean age of 14.46 ± 9.44 years (range: 0.4-47 years) and an increase in males (80/136, 58.8%). Body Mass Index (BMI) was calculated for each patient; 16.7% (20/120) were underweight (BMI <18.5 kg/m^2 for adults and <p5 for children), 47.5% (57/120) were classified in the normal range (18.5-24.5 kg/m^2 for adults and p5-p85 for children), 15.8% (19/120) were classified as overweight (25-30 kg/m^2 for adults and p85-95 for children) and 20% (24/120) were classified in the obesity range (>30 kg/m^2 [f] for adults and >p95 for children). For comparison with other variables, patients with a BMI in the range of underweight and normal were grouped and compared with patients with BMI in the overweight and obesity range. There were no statistical differences in age or BMI between genders.

As for the molecular characterization of the deletion, 89.7% (122/136) have a 1.55 Mb deletion and 10.3% (14/136) have a 1.83 Mb deletion. The parental origin of the deletion was in 50.7% (69/136) of maternal origin. An inversion-mediated deletion was present in 29.5% (36/122) of cases. *NCF1* copy number was studied in all but one patient of our cohort due to bad quality DNA. Of the patients studied, 49.6% (67/135) had one copy, 41.5% (56/135) had 2 copies and 8.8% (12/135) had three or four copies. Depending on the location of the breakpoint, WBS patients can lose a copy of *NCF1* gene and can also gain copies secondary to gene conversion events [21, 53].

Cardiovascular lesions

In the medical reports, 78.7% (107/136) of patients presented with a cardiovascular lesion (Table 2), the two most frequent being the supravalvular aortic stenosis (SVAS) and the peripheral pulmonic stenosis (PPS). Valvular disease was also very frequent in this cohort, mitral valve prolapse (MVP) (8/136), mitral valve insufficiency (9/136) and stenosis (1/136), aortic valve insufficiency (6/136) and stenosis (1/136) and pulmonary valve insufficiency (2/136). Surgical treatment was performed in 26% (25/96) of this cohort.

Cardiovascular lesion	Medical history information (n=136)	Echocardiographic findings (n=47)
SVAS	59.6% (81/136)	46.8% (22/47)
PPS	39.7% (54/136)	17% (8/47)
Aortic coarctation	5.9% (8/136)	10.6% (5/47)
Valvular disease	15.4% (21/136)	31.9% (15/47)

Table 2. Cardiovascular lesions reported in the cohort of 136 patients and in the 47 patients who underwent an echocardiographic study.

In the cohort of 47 patients with an echocardiogram performed, about 80.8% of patients presented a cardiovascular lesion. More than one cardiovascular lesion was present in 55.3% (21/38) of cases. PPS was diagnosed in a smaller percentage, while aortic coarctation and valvular disease were present in higher numbers. As in the general cohort, the most frequent valvular disease were MVP (10.6%, 5/57), mitral insufficiency (12.8%, 6/47), aortic valve insufficiency (6.4%, 3/47). Surgical treatment was performed in 14.9% (7/47) of patients.

A carotid Doppler US was performed and 13.8% (4/29) presented carotid arteries with a prominent intima but normal lumen. A supraaorctic and abdominal Doppler US was also performed in the cohort of 47 patients. An alteration was reported in 42.5% (20/47) of cases and included abdominal aorta stenosis (19.1%, 9/47), celiac trunk stenosis (6.4%, 3/47), superior mesenteric artery (SMA) stenosis (4.3%, 2/47) and renal artery stenosis (2.1%, 1/47). In fourteen patients, a CT angiogram was also performed. Patients with alterations reported by Doppler US also had alterations reported in the CT angiogram. However, 9 of the 27 patients with a normal Doppler US also had a CT angiogram performed. Of these 9 cases, only 2 cases had normal results and the remaining 7 presented luminal narrowing of the abdominal aorta (6/7), middle aortic syndrome (4/7), celiac trunk stenosis (5/7) and superior mesenteric artery stenosis (2/7). Six of the seven patients presented more than one lesion.

Male patients presented a higher frequency of cardiovascular lesions (86.3%, 69/81) compared to female patients (67.9%, 38/56) ($p=0.010$). Male patients also presented a more severe cardiovascular disease (30.4%, 21/69) compared to female patients (13.2%, 5/38) ($p=0.046$). There was no significant difference in the prevalence of SVAS and PPS between genders.

Patients with cardiovascular disease were significantly younger (12.83±8.28

years) when compared to patients without lesions (20.48±11.11 years) ($p<0.001$). The comparison was also significant for PPS (12.01±7.89 years vs. 16.08±10.08 years) ($p=0.018$) and SVAS (12.52±7.06 years vs. 17.34±11.11 years) ($p=0.020$).

Cardiovascular lesions and their severity were not associated with BMI.

Office and Ambulatory Blood Pressure Monitoring Arterial Hypertension

Based on office BP measurements, 52.2% (71/136) had systolic HTN and 42.6% (58/136) had diastolic HTN. Pharmacological treatment was reported in 30.6% (34/111) of cases. Some of the drugs used were angiotensin II receptor blockers (11/34), β-blockers (7/34), angiotensin converting enzyme (ACE) inhibitors (5/34), calcium channel antagonists (4/34) and diuretics (2/34). Three patients required more than one drug to control their HTN.

Systolic HTN was significantly more frequent in patients with a BMI in the overweight and obesity range compared to patients with BMI in the normal range ($p=0.020$) (Figure 1). Diastolic HTN was also more frequent in overweight and obese patients but did not reach statistical significance ($p=0.056$).

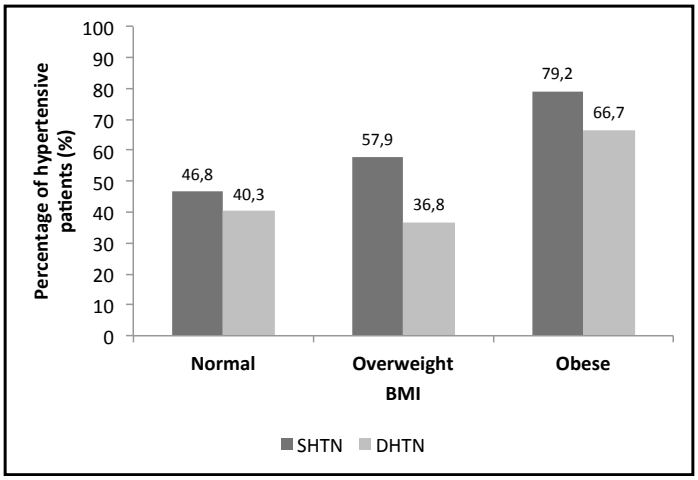

Figure 1. Percentage of patients with HTN compared to BMI classification.

Twenty-four hour ABPM was performed in a cohort of 49 individuals with a mean age of 16.2±10.5 years (with a range of 0.8-44 years) and with slightly higher frequency of females (57.1%, 28/49). BMI was also calculated for this cohort, 58.3% (28/48) presented an index within the normal range, 10.4% (5/48) were in the overweight range and 31.3% (15/48) were in the obesity range. Before the study, only 18.4% (9/49) patients had been diagnosed with HTN.

The mean values of the ABPM variables studied are presented in Table 3. We decided to take into account only ABPM with 7 or more diurnal reliable readings and 4 or more nocturnal readings.

	Mean ± SD (n=42)	Normotensive (n=28)	Hypertensive (n=14)
Age (years)	17,66 ± 10,62	15.84 ± 11.02	18.57 ± 10.51
Diurnal readings	16,88 ± 10,87	17.25 ± 11.72	16.14 ± 9.30
Nocturnal readings	7,17 ± 2,66	7.07 ± 2.72	7.36 ± 2.62
HR (bpm)	85,5 ± 11,4	83.57 ± 13.08	89.54 ± 4.31
BPI	0,982 ± 0,097	0.928 ± 0.061	1.09 ± 0.056
SBPI	0,998 ± 0,094	0.948 ± 0.061	1.099 ± 0.061
DBPI	0,953 ± 0,107	0.895 ± 0.071	1.069 ± 0.017
Nocturnal BPI	1,019 ± 0,111	0.975 ± 0.091	1.106 ± 0.099
SBPL daytime (%)	40,81 ± 33,38	23.14 ± 20.61	76.16 ± 24.76
SBPL nighttime (%)	52,60 ± 32,44	40.13 ± 28.59	77.55 ± 24.83
DBPL daytime (%)	28,79 ± 26,71	14.12 ± 14.21	58.13 ± 21.01
DBPL nighttime (%)	44,91 ± 34,03	33.79 ± 31.74	67.16 ± 27.55
Systolic SD daytime (mmHg)	13,11 ± 4,37	12.99 ± 3.84	13.37 ± 5.43
Systolic SD nighttime (mmHg)	12,38 ± 4,77	12.13 ± 4.30	12.88 ± 5.73
Diastolic SD daytime (mmHg)	10,43 ± 3,02	10.46 ± 3.06	10.37 ± 3.06
Diastolic SD nighttime (mmHg)	9,49 ± 4,12	9.38 ± 4.25	9.70 ± 3.99
24h-PP (mmHg)	51,42 ± 7,71	49.51 ± 6.44	55.24 ± 8.83
Diurnal PP (mmHg)	52,27 ± 9,14	49.88 ± 6.81	57.04 ± 11.42
Nocturnal PP (mmHg)	49,73 ± 7,69	48.33 ± 7.41	52.53 ± 7.75
AASI	0,451 ± 0,187	0.417 ± 0.153	0.518 ± 0.231

Table 3. ABPM variables studied in a cohort of 49 patients with WBS. BPI (blood pressure index), SBPI (systolic blood pressure index), DBPI (diastolic blood pressure index), SBPL (systolic blood pressure load), DBPL (diastolic blood pressure load), SD (standard deviation), PP (pulse pressure) and AASI (ambulatory arterial stiffness index).

After 24-hour ABPM, 33% (14/42) had a MBP above the 95th percentile, 42.9% for SBP (18/42), 31% for DBP (13/42) and 57.1% (24/42) for nocturnal BP. We evaluated the frequency of isolated systolic, diastolic and nocturnal HTN in our cohort. Isolated systolic HTN, defined as SBP above the 95th percentile with a MBP below the 95th percentile for age and gender was present in 9.5% (4/42) of patients. Isolated diastolic HTN, defined as a DBP above the 95th

percentile with MBP below the 95th percentile for age and gender, was present in 2.4% (1/42) of patients. Isolated nocturnal HTN, defined with a nocturnal BP above the 95th percentile with a MBP below the 95th percentile for age and gender, was present in 28.6% (12/42).

Severe ambulatory HTN, defined as having more than 50% of readings above the 95th percentile, was found in 40.4% (17/42) for diurnal mean SBP, in 23.8% (10/42) for diurnal mean DBP, in 57.1% (24/42) for nocturnal mean SBP and in 50% (21/42) for nocturnal mean DBP.

Circadian variation was evaluated using the dipping status; the mean value of our cohort of WBS patients was in the non-dipper category (Table 3), with a nocturnal BP reduction of less than 10% when compared to diurnal BP measurements. Non-dippers were 42.9% (18/42) of patients in our cohort. However, 35.7% (15/42) of patients were classified as dippers, with a nocturnal BP reduction between 10-20% with respect to diurnal BP, and 9.5% (4/42) were classified as inverse dippers with a nocturnal BP increase with respect to diurnal BP. Extreme dippers were 11.9% (5/42) of patients, with a reduction of nocturnal BP above 20% with respect to diurnal. Approximately 40% (17/42) patients with MBP below the 95th percentile, in the normal range presented an abnormal dipping status.

Adult hypertensive patients had significantly increased 24h-PP (60.88 ± 7.98 vs. 51.17 ± 4.22 mmHg, p=0.003), daytime PP (63.94 ± 11.4 vs. 51.36 ± 5.73, p=0.007) and nocturnal PP (56.73 ± 7.11 vs. 50.18 ± 4.86, p=0.034). AASI was higher in adult hypertensive patients but was not significant. Although not significant, hypertensive pediatric patients also presented a higher PP and AASI compared with normotensive patients.

There were no statistical differences by gender in the frequency of HTN, BPI or dipping status. However, male patients presented significantly higher nocturnal SBPL and DBPL (64.6 ± 26.8%; 58.1 ± 27.9%) compared to female patients (43.6 ± 33.9%; 35.0 ± 35.4%) (p=0.026, 0.027; respectively). Male patients also presented greater nocturnal systolic and diastolic BP variability compared to female patients (p=0.018 and 0.007, respectively).

We also analyzed if patients presenting HTN were significantly older than normotensive patients and there was no significant age difference between both groups. Pediatric patients presented a similar proportion of mean, systolic, diastolic and nocturnal HTN compared to adults (Table S1). Age was significantly correlated with 24-hour PP (r(41)=0.412, p=0.007), diurnal PP (r(41)=0.384, p=0.012) and nocturnal PP (r(41)=0.416, p=0.006).

There were associations between BMI with some ABPM variables: an increased BMI was associated with increased BPI, SBPI and DBPI compared to patients with a BMI in the normal range (Figure 2). The mean BPI´s are all above 1 (>95th percentile) in the case of overweight and obese individuals, while the mean values of individuals with a BMI in the normal range are all below 1. The prevalence of HTN was also significantly increased in

patients with a BMI in the obesity (53.8%, 7/13) and overweight range (60%, 3/5) compared to patients with a BMI in the normal range (13%, 3/23) (p=0.014).

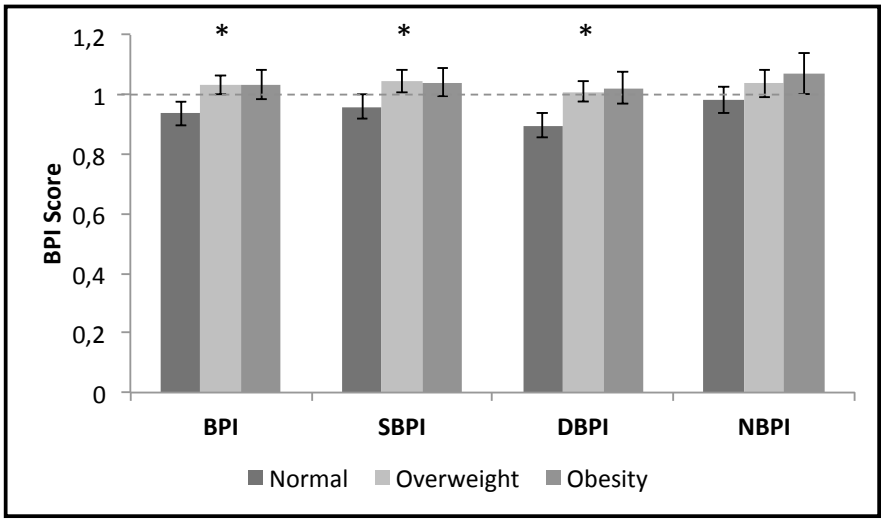

Figure 2. Mean, systolic and diastolic BPI compared by BMI classification. Values above 1 are defined as hypertensive, corresponding to values above the 95th percentile (dotted line). Patients with BMI in the overweight range had significantly increased BPI (p=0.003), SBPI (p=0.017), DBPI (p<0.001) and nocturnal BPI (p=0.061) when compared to patients with BMI in the normal range. Asterisks denote statistical significance with a p<0.05.

Left ventricle hypertrophy and morphology

Left ventricle hypertrophy (LVH), defined as having a left ventricle mass (LVM) index ($g/m^{2.7}$) above the 95th percentile for age and gender, was present in 25.5% (12/47) of WBS patients and with extreme values above 51 $g/m^{2.7}$ in 17% (8/47) of patients.

LV geometry was determined using the LVM index ($g/m^{2.7}$) and the relative wall thickness. LV geometry was abnormal in 59.6% (28/47) of WBS patients; specifically 34% (16/47) had concentric remodeling of the LV, 19.1% (9/47) had concentric hypertrophy and 6.4% (3/47) had eccentric hypertrophy.

There was no significant difference in LVH or alterations in LV geometry with respect to gender or age.

When compared by BMI, patients with high BMI had increased frequency of LVH (60%, 9/15) compared with patients with a BMI in the normal range (18.8%, 6/32) (p=0.008). Patients with increased BMI also presented with increased frequency alterations in LV geometry (p=0.001) as shown in Figure 3.

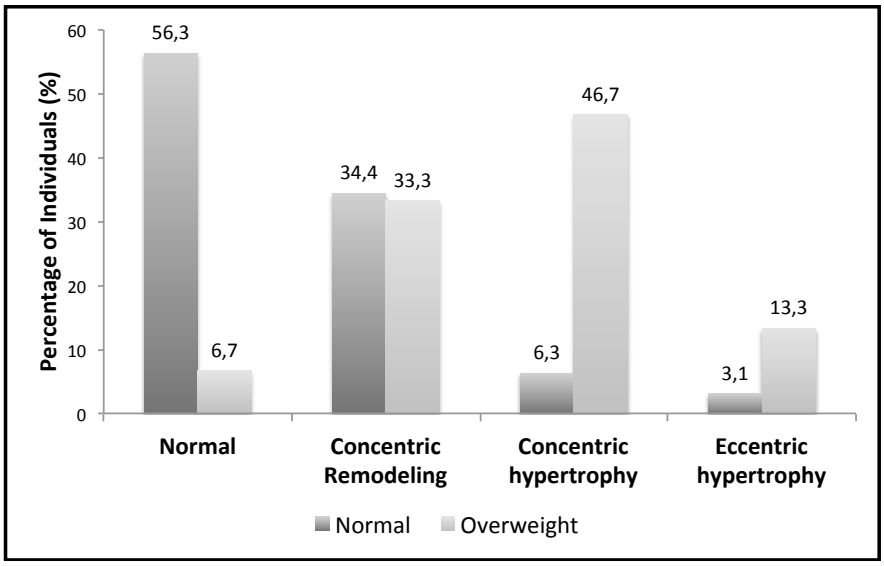

Figure 3. Percentage of patients with LV geometry alterations separated by groups of BMI.

RAS

Plasma renin activity (PRA) was available for 47 patients and aldosterone plasma levels for 43 patients. There was no significant difference in PRA or aldosterone in hypertensive (1.74 ± 0,97, 15.60 ± 9,14) and non-hypertensive patients (1.87 ± 1.70, 17.71 ± 12.4). There were no statistical associations when comparing PRA or aldosterone with 24-hour mean blood pressure or BPI´s.

There was also no association of PRA and aldosterone plasma concentrations with age or gender. However, patients with a BMI in the overweight range had lower PRA values (1.10 ± 0.773) compared to patients with a normal BMI (2.34 ± 1.68) (p=0.020).

Oxidative Stress

Oxidative stress parameters were measured in 32 WBS patients from Hospital Vall d´Hebron. MDA plasma concentrations had a normal distribution (Supplementary Figure S2). Higher levels of MDA were observed in the hypertensive group, for mean, systolic, diastolic and nocturnal BP, as shown in Figure 4. Patients with two or more *NCF1* copies had increased MDA plasma concentration compared to patients with only 1 copy, although it did not reach statistical significance (Figure S3). Five patients had extreme values (considered above 0.63 μM for our cohort) and four of them had two or more *NCF1* copies.

There were no significant differences when compared by age, gender or BMI.

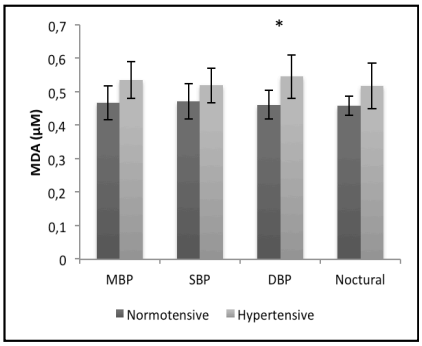

Figure 4. MDA concentration in normotensive and hypertensive individuals (*p=0.05).

MDA concentration was not associated with any other ABPM or echocardiographic variable studied.

There was no significant difference between the remaining of oxidative stress markers measured and mean, systolic, diastolic and nocturnal blood pressure (Table S2) or with *NCF1* copy number.

Ambulatory Hypertension - Losartan treatment

Eight patients with MBP above the 95th percentile for age and gender received losartan at a dose of 1 mg/kg/day and were followed-up one year later. ABPM was not available for one patient at 12-months after treatment due to monitoring interference. As shown in Figure 5A, there was a significant reduction in mean, systolic, diastolic and nocturnal BPI after 12 months of treatment.

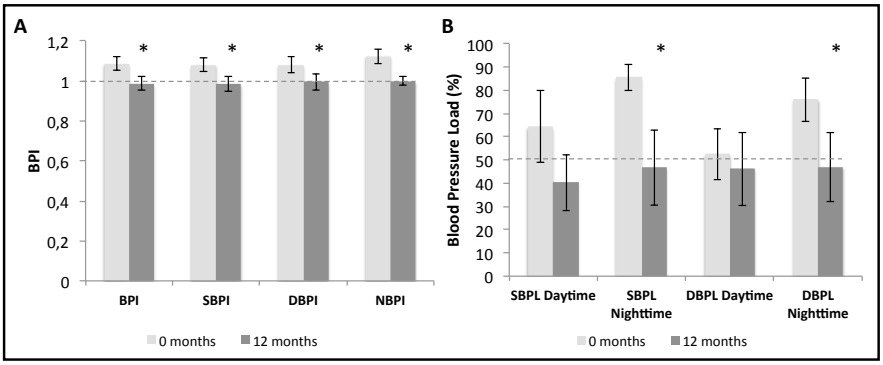

Figure 5. A. Mean, systolic, diastolic and nocturnal BPI at 0 months and 12 months after losartan treatment (*p=0.018). Values above 1 are defined as HTN, corresponding to values above the 95th percentile (dotted line). **B.** Systolic and diastolic blood pressure loads at daytime and nighttime before and after losartan treatment. The dotted line represents the percentage of readings for severe ambulatory hypertension (*p=0.028).

There were also significant reductions in nocturnal systolic and diastolic blood pressure loads (Figure 5B), but not on diurnal blood pressure loads. Although not significant, diurnal blood pressure loads also decreased. At 12 months all blood pressure loads were below the cut-point of severe ambulatory blood pressure (50% of readings above the 95th percentile).

There were no significant reductions in blood pressure variability. Although not significant, pulse pressure and AASI

decreased after 12-months of treatment with losartan (Table S3).

PRA and aldosterone levels were measured at the initial visit and at 12 months. As observed in Figure 6, after 12 months of treatment PRA and aldosterone levels increased.

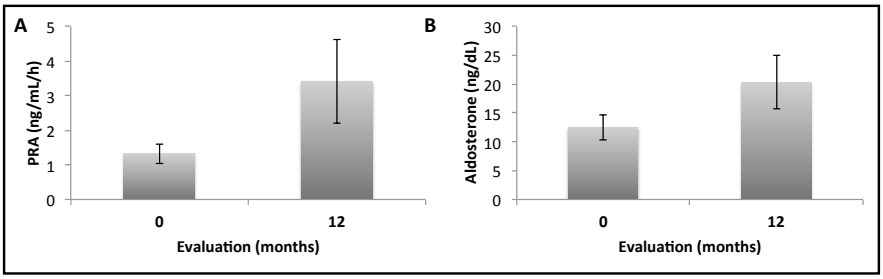

Figure 6. A. PRA at 0 and 12-month follow-up. PRA increased after 12-months of treatment with losartan (p=0.063). **B.** Aldosterone plasma levels after 12-month follow-up. (PRA: Plasma Renin Activity).

Reduction in oxidative stress variables after treatment with losartan was also analyzed. There was a significant reduction in MDA plasma levels after 12 months of treatment (Figure 7). There were no significant changes with the other markers studied (Table S3).

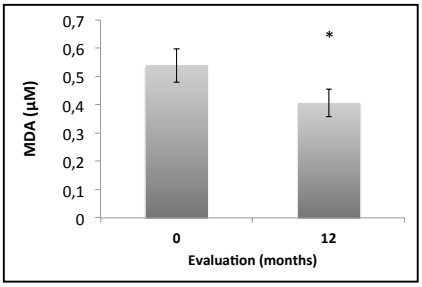

Figure 7. MDA concentration at 0 and 12 months after losartan treatment (p=0.017).

Clinical correlations

Ambulatory HTN was compared with the number of vascular stenoses detected by Doppler US and CT angiogram. There was no difference in the frequency of vascular stenosis detected by Doppler US between normotensive and hypertensive patients (Table 4). However, hypertensive patients presented a higher mean number of stenosis (0.77 stenosis per patient) compared to normotensive (0.17 stenosis per patient). It should be taken into account that 13 patients did not agreed to have an ABPM performed and were not included in this analysis.

	Doppler US		CT Angiogram	
	Normotensive (n=24)	Hypertensive (n=9)	Normotensive (n=8)	Hypertensive (n=6)
Abdominal aorta stenosis	2	3	1	0
Diffuse narrowing of abdominal aorta	-	-	3	2
Celiak trunk stenosis	1	2	6	3
Superior mesenteric artery stenosis	1	1	3	3
Renal artery stenosis	0	1	0	0
Middle aortic syndrome	-	-	3	3
Mean number of lesions	0.17	0.77	1.63	1.77

Table 4. Frequency of vascular stenosis detected by Doppler US and CT angiogram in normotensive and hypertensive individuals with WBS.

In regards to CT angiogram, there were no difference between the frequency of vascular stenosis and the mean number of lesions between normotensive and hypertensive individuals. It should be mentioned that 7 of the normotensive individuals had a MBP between the 75th and 95th percentile. The only patient with a MBP below the 75th percentile had no vascular stenosis detected by CT angiogram.

We also compared our ABPM variables with LVH and LV geometry to determine whether HTN or cardiovascular lesions could have an effect on heart structure. LVH was also associated with HTN, 67% (6/9) hypertensive patients presented increased LV mass compared to 10.5% (2/19) of normotensive individuals (p=0.005). This association was significant for mean, systolic (p=0.030), diastolic (p=0.005) and nocturnal hypertension (p=0.002).

With regards to LV geometry, patients with eccentric or concentric hypertrophy presented higher BPI compared to patients with normal LV geometry with a mean value above 1, which corresponds to the 95th percentile (Figure 8). Patients with concentric remodeling presented higher BPI compared to the normal geometry group but it did not reach 1. There were also significant differences with respect to diurnal systolic (p=0.013) and diastolic (p=0.021) BPL and nocturnal diastolic BPL (p=0.013). Patients in the concentric hypertrophy group had the highest percentages of readings above the 95th percentiles compared to the other three groups.

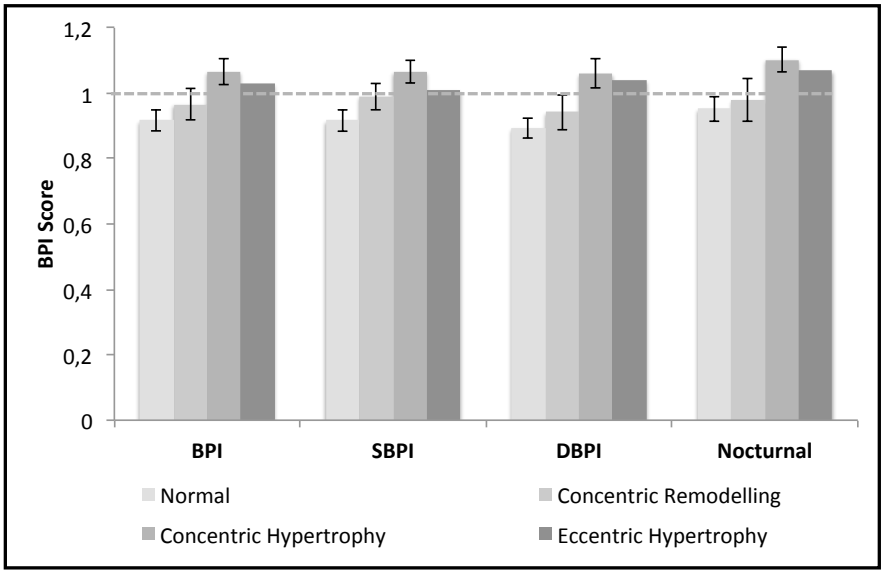

Figure 8. Mean, systolic, diastolic and nocturnal BPI and LV geometry. There was a significant association in BPI (p=0.009), SBPI (p=0.004), DBPI (p=0.008) and nocturnal BPI (p=0.031).

Although some patients with vascular stenoses (including SVAS) presented LVH or an abnormal LV geometry, there was no significant association among the presence of vascular stenoses and LVH or an abnormal LV geometry.

Molecular correlations

We found no significant association between *NCF1* copy number and cardiovascular disease. Among the vascular anatomic findings in terms of significant stenosis observed on echocardiogram, Doppler US or Vascular CT celiac trunk stenosis were only present in patients with two or more *NCF1* copies (17,4% 4/23 vs. 0%, 0/24) (p=0.05), there were no significant genotype differences for other vascular lesions.

There was no significant association of NCF1 copy number with office and ambulatory HTN. Although it did not reach statistical significance; when subdivided by gender, 32.1% (9/28) of female patients with one *NCF1* copy presented office HTN compared to 58.3% (14/24) female patients with two or more *NCF1* copies (p=0.058) (Figure 9).

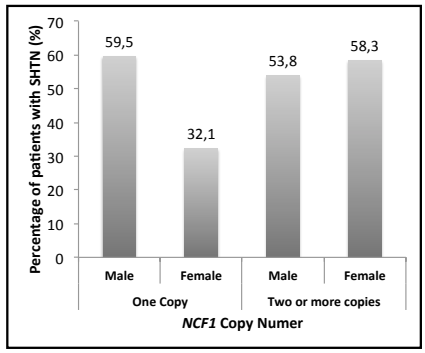

Figure 9. *NCF1* copy number and percentage of office systolic hypertension subdivided by gender (in females, p=0.058).

We searched for an association between cardiovascular lesions and office HTN with the nine-selected candidate SNPs. Relevant SNPs and the associations are shown in Table 5. We found a significant difference in allelic frequencies of *PLCE1* gene (p=0.032), *ELN* gene (p=0.0145) and *AGT* gene (p=0.0015) between patients with cardiovascular stenoses and those without (Table 5). Allelic and genotypic frequencies of angiotensin converting enzyme (*ACE*) gene were significantly different between patients presenting PPS from patients without (p=0.036, p=0.013, respectively). Fibulin 2 (*FBLN2*) genotypic (p=0.007) and allelic frequencies (p=0.026) were also significantly different between patients with valvular alterations compared to those patient without. *CACNB2* and *PLCE1* gene were also significantly associated with valvular disease (Table 5).

Gene (SNP)	Association	Genotype	HapMap CEU Freq	Controls (Freq)	Cases (Freq)	OR (95CI)	P-value
ELN **(rs2071307)**	Cardiovascular alterations	A	0.385	0.640 (16)	0.360 (9)	3.167 (1.257-7.981)	0.0145
		G	0.615	0.360 (32)	0.640 (57)		
PLCE1 **(rs932764)**	Cardiovascular alterations	A	0.572	0.760 (38)	0.593 (115)	1.81 (1.14-2.87)	0.0117
		G	0.428	0.240 (12)	0.407 (79)		
AGT (rs7079)	Cardiovascular alterations	A	0.327	0.533 (32)	0.304 (59)	0.382 (0.212-0.691)	0.0015
		C	0.673	0.467 (28)	0.696 (135)		
FBLN2 **(rs3732666)**	Valvular disease	A	0842	0.809 (165)	0.650 (26)	2.278 (1.09-4.76)	0.029
		G	0.158	0.191 (39)	0.350 (14)		
ACE (rs4305)	PPS	A	0.435	0.563 (81)	0.400 (40)	0.519 (0.309-0.871)	0.013
		G	0.565	0.437 (63)	0.600 (60)		
CACNB2 **(rs11014166)**	Valvular disease	A	0.633	0.672 (137)	0.475 (19)	2.26 (1.138-4.488)	0.0198
		T	0.367	0.328 (67)	0.525 (21)		
PLCE1 **(rs932764)**	Valvular disease	A	0.572	0.593 (121)	0.800 (32)	0.365 (0.16-0.83)	0.016
		G	0.428	0.407 (83)	0.200 (8)		

Table 5. Association of relevant SNPs with cardiovascular stenoses and valvular disease.

There was also a tendency for an association between office systolic and diastolic blood pressure and *PLCE1* allelic frequency (Table 6). Both the SBPI (p=0.019) and DBPI (0.017) increase significantly with each G-allele in an additive model (Figure 10).

Gene (SNP)	Association	Genotype	HapMap CEU Freq	Controls (Freq)	Cases (Freq)	Literature (Beta score)	P-value	Controls (Freq)	Cases (Freq)	OR (95CI)	P-value
***PLCE1* (rs932764)**	SHTN	A	0.572	0.56	0.500	G: 0.484	SBP $7.1x10^{-16}$	0.683 (82)	0.573 (71)	1.611 (0.954-2.72)	0.074
		G	0.428	0.44	0.500			0.317 (38)	0,427 (53)		
***PLCE1* (rs932764)**	DHTN	A	0.572	0.56	0.500	G: 0.484	SBP $7.1x10^{-16}$	0.674 (97)	0.560 (56)	1.622 (0.958-2.75)	0.071
		G	0.428	0.44	0.500			0.326 (47)	0.440 (44)		

Table 6. Association of *PLCE1* gene with systolic and diastolic HTN.

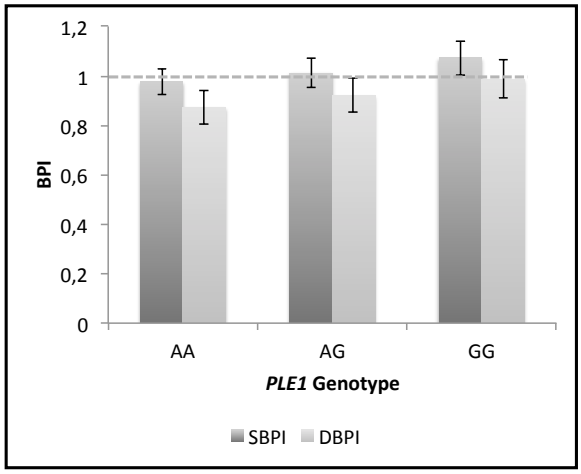

Figure 10. Association of SBPI and DBPI with *PLCE1* genotype.

DISCUSSION

In this study we have compiled and analyzed information about cardiovascular alterations and HTN from 136 patients with WBS. We have also analyzed the results of echocardiography and ABPM in a smaller cohort of patients. Hypertensive patients detected with ABPM were prescribed an angiotensin II receptor blocker and performed a control after 12-months of treatment. Finally, we have included the analysis of the renin-angiotensin system and oxidative stress markers, and tried to search for possible genetic modifiers of the cardiovascular phenotype (including *NCF1* gene copy number and previously described candidate SNPs for HTN).

The frequency of cardiovascular alterations was very similar between the information obtained from the medical reports and the results of the echocardiogram in the smaller cohort. The frequency of cardiovascular alterations was also comparable to what has been previously published in the literature. A review of 9 international

series reported that approximately 84% (53-100%) of patients with WBS present a cardiovascular alteration [14]. As in other studies, the two most frequent cardiovascular alterations were SVAS (47-52%) and PPS (17-35.4%). The incidence of PPS is more variable, since it depends on the age of the cohort, as it tends to improve and eventually resolve with age [54, 55]. Valvular disease was also a frequent alteration in our cohort, the most frequent alterations being MVP and aortic or mitral valve insufficiency. The mitral valve is trilaminar and each layer has unique characteristics and biomechanical properties fundamental for its proper function [56]. The atrialis layer is rich in subendothelial elastic proteins that mitigate leaflet stretch during systole [56]. Therefore, the decrease and abnormal production of elastin content in WBS patients might explain why the leaflets do not cover sufficiently the mitral annulus orifice causing the insufficiency. MVP, also present in patients with connective tissue syndromes (such as Ehlers-Danlos syndrome), has been associated with collagen, elastin and proteoglycan deficiency [56].

Approximately 20% of our cohort underwent surgical management, which is also in concordance with other studies [57].

We found that 13.8% of patients presented an increase in intima-media wall thickness of the carotid arteries. Although our sample number was low, we did not find any significant relationship with age, gender or HTN. Increased media thickness of carotid arteries has been previously described in WBS patients and it shows the generalized presence of WBS arteriopathy [58].

Approximately 43% of our cohort presented alterations in the abdominal aorta detected by Doppler US, including stenosis of the descending aorta, celiac trunk (CT), superior mesenteric artery (SMA) and renal artery. In 14 patients we also studied the results of a CT angiogram. Patients with anomalies detected by Doppler US were corroborated by CT angiogram, which identified other alterations in some cases. Among the 9 patients with a normal Doppler US, only 2 had a normal CT angiogram, the remaining 7 patients presented stenoses of the SMA or CT or middle aortic syndrome. A study in 25 individuals with WBS found that 80% (20/25) had vascular stenosis of the abdominal aorta detected by an aortography [59]. This detection rate is similar to ours when using a CT angiogram. This corroborates that cardiovascular disease in WBS is characterized by stenoses of medium and large-sized arteries [14], and that any artery can be affected. Middle aortic syndrome is characterized by narrowing of the descending aorta with stenosis of the renal and other main arteries [60]. Its prevalence in WBS is uncertain, with a range of 2-70% [57, 61, 62]. In our cohort, 57% of patients studied by CT angiogram had middle aortic syndrome. The greater detection rate (particularly of intrathoracic stenoses) and greater precision (greater number of clearly visualized abdominal branch stenoses) of CT angiogram over Doppler US justify the choice for diagnosis and follow-up, despite of radiation risks.

MRA has a similar or greater detection rate and avoids radiation but its availability and cost could not make it the first choice in our case. Some of the described complications of abdominal artery stenoses include renovascular hypertension and portal hypertension [63, 64]. The detection of abdominal vascular stenosis usually occurs after patients have presented significant symptomatology. Therefore, it is important when evaluating a patient with WBS with this symptomatology to keep in mind these possible causes in order to diagnose and treat promptly.

We found a significant increased prevalence of cardiovascular disease and severity in male patients compared to female patients. This has previously been reported and it has been postulated that the difference could be caused by sex hormones, since they are known to be important regulators of cardiovascular development and function [65].

HTN is also a very common clinical manifestation in WBS. Its frequency varies largely, between 5-59%, depending on the criteria used, referral bias, age variation and measurement methodology used (sphygmomanometer or ABPM) [9]. In essential HTN many pathophysiologic factors have been implicated, including increased sympathetic activity, overproduction of vasoconstrictors and sodium-retaining hormones, increased secretion of renin, vasodilator deficiency, alterations in vessel resistance, oxidative stress, among others [66].

The causative mechanism of HTN in WBS has not been established yet. It is known that the cardiovascular manifestations in WBS are caused by the deletion of the elastin gene. Elastin is the main component of the arterial extracellular matrix [67] and its haploinsufficiency leads to endothelial cell proliferation and smooth-muscle reorganization causing luminal narrowing [68]. Therefore, it is likely that HTN in WBS patients could be secondary to an increased arterial stiffness and alterations in the distensibility of the aorta and systemic arteries [6]. The increased cardiac afterload would explain the increase in blood pressure. In our cohort, approximately 52% of patients presented systolic and 43% diastolic office HTN.

ABPM analysis in 47 patients with WBS revealed that 33% of patients had HTN, which is a little lower than what has been reported previously (≈40%) [8, 9]. As in these two studies, systolic HTN (53%) was more frequent than diastolic HTN (31%) [8, 9]. We also found that 9.5% of patients presented systolic and, 2.4% diastolic isolated HTN. Isolated systolic HTN prevalence was higher (2.7-5.8% in children), while diastolic HTN was similar to what has been reported in the general population (3.6% in adults) [69, 70]. Isolated systolic HTN in older adults is associated with increased arterial stiffness most often secondary to atherosclerosis [71]. In children, it has been suggested to be secondary to sympathetic nervous system hyperactivity [71]. Morphologic alterations in blood vessels secondary to elastin deficiency and arterial stiffness are present from childhood in WBS;

therefore, arterial stiffness could also explain the increased prevalence of systolic HTN in this cohort. Increased arterial stiffness and decreased compliance in WBS children has been previously reported [72]. The authors also reported a strong correlation between SBP and arterial stiffness[72].

PP and AASI indirectly assess arterial stiffness and have been associated with target-end organ damage and cardiovascular mortality in adults [73, 74]. In children and adolescents, PP appears to be more closely associated with arterial stiffness and target end-organ damage compared to AASI [75]. Adult hypertensive patients with WBS presented significantly higher 24-h, daytime and nighttime PP compared to normotensive patients. Although not significant, pediatric hypertensive patients also presented higher PP compared to normotensive patients. This is also in concordance that HTN in WBS could be associated with increased arterial stiffness.

The most frequent symptom associated with middle aortic syndrome is HTN [60]. We did not find a significant association between vascular stenoses and HTN. Nevertheless, hypertensive patients had a higher number of abdominal artery stenosis per patient compared to normotensive patients when compared by US. Although our sample size may be too small for the CT angiogram to detect an association with HTN, the only patient with a MBP below the 75th percentile did not present any vascular stenosis. We describe one patient with renovascular hypertension (renal artery stenosis). As previously reported, in few cases HTN is secondary to renovascular disease and HTN in WBS should be regarded as a manifestation of generalized arteriopathy rather than renal hypoperfusion [76]

We found a significant proportion of individuals with nocturnal HTN (57%) in comparison to other studies. Approximately 29% of patients presented nocturnal isolated HTN, a much higher frequency compared to adults in the general population (7%) [27]. Several studies in adults have shown that nocturnal HTN in an independent risk factor associated with end-organ damage and cardiovascular prognosis [77, 78]. In children with chronic kidney disease and diabetes mellitus nocturnal HTN has been found to have significant prognostic implications [79, 80].

Blunted nocturnal BP has also been independently associated with increased cardiovascular morbidity and mortality [81, 82]. The proportion of non-dippers in our hypertensive group was approximately 57%, a little higher than the range (25-40%) described in the general population and in a previous study in patients with WBS (47%, 8/17) [10, 83]. However, it is more striking the proportion of altered dipping patterns in the normotensive group (approximately 40%). In one of the studies of APBM in WBS patients, the authors also observed a blunted nocturnal dip in normotensive subjects [8]. A recent study using ABPM found that WBS patients had increased nocturnal heart rate and impaired reduction during the night compared to controls [84]. Alterations in circadian variation have been associated with an

abnormal activation of the sympathetic activity and with sleep disturbances [83, 84]. Several studies report that adults and children with WBS present behavior patterns associated with sleep disturbances, such as daytime sleepiness, fragmented sleep or night waking [85, 86]. Therefore, sleep disturbances and increased sympathetic activity could be the case of nocturnal HTN and blunted nocturnal decrease in WBS patients.

BPL is a better predictor of end-organ damage than mean BP [87] and it has been shown in children that when BPL is above 50% (severe ambulatory hypertension) it can predict LVH [88]. In our cohort, we had an important number of patients with severe diurnal (50%) and nocturnal (50%) ambulatory HTN who presented LVH. These patients should be monitored to control LVH and possible future complications.

We did not find a significant association between ambulatory HTN and plasma renin activity or aldosterone levels. As in essential HTN, plasma renin levels varied widely among patients [89].

It has been thought that hypertensive disease in WBS was more frequent in adult patients [9, 59]. However, as in the other ABPM study [8], we found no significant association with age. Children (younger than 18 years) presented a similar frequency of mean, systolic, diastolic and nocturnal hypertension compared to adult patients (older than 18 years). The same proportion of hypertensive children had LHV compared to adults. The only difference between both groups was the prevalence of increased BMI (67% in adults compared to 26% in children). This shows that HTN in WBS is similar to essential hypertension but more severe, with a younger age of presentation and that obesity seems to play a greater role in adults than in children with WBS. Due to the complications of HTN observed in adults and children with WBS [90–92], it is very important to control office BP irrespective of age and symptomatology.

The prevalence of overweight and obesity in our cohort was 10-16% and 20-31%, respectively. This frequency is similar to the rate that has been previously published in adolescents and adults with WBS [93, 94]. As in the general population and in WBS [8], we also found a significant association of office and ambulatory HTN with an increased BMI. The mechanisms linking obesity and HTN are not completely understood. However, it has been thought that obesity causes HTN by an activation of the sympathetic nervous system, impairment of pressure natriuresis, increased plasma renin activity and aldosterone levels, structural changes in the kidney, hormonal alterations and endothelial dysfunction [95]. The underlying mechanisms associating obesity and HTN in WBS are also unknown. We also found a significant association between LVH and BMI, which is in concordance with previous studies that have described an association between LVM and lean body mass, fat mass, gender and SBP in children [96]. The impact of LVH and abnormal LV geometry in WBS patients is still unknown, however they are known to be sensible markers of target damage in

children and adults [96, 97]. Due to the high frequency of cardiovascular risk factors (vascular stenosis, HTN, glucose intolerance) in WBS it is highly recommended to have a BMI within the normal range by maintaining a well-balanced diet and regular physical activity.

LVH was found in 25.5% of patients with WBS. A much smaller frequency compared to a previous study (56%). However, their population number was smaller (n=16) [11]. Nevertheless, we corroborate their findings, significant LVH may develop from childhood [11]. We found a significant association between mean, systolic, diastolic and nocturnal HTN with LVH. This corroborates that ABPM has a high correlation with LVH [88, 98]. Although not statistically significant, some of the patients presenting vascular stenoses also presented LVH. Nevertheless, it might seem that in this cohort the principal factor associated with LVH was HTN.

LV geometry was abnormal in approximately 60% of our cohort, which is similar to what has been reported previously [11]. All hypertensive patients in our cohort presented an abnormal left ventricular geometry. Patients without HTN but with cardiovascular lesions (SVAS, PPS, coarctation of the aorta or MVP) also presented alterations in LV geometry. There was one patient with LV concentric hypertrophy without cardiovascular lesions or HTN. This is in concordance with a previous study that also detected textural abnormalities in the absence of SVAS or HTN [11]. It seems that LV geometry may add prognostic information to the evaluation of LVH alone in the assessment of hypertensive patients [99].

Although infrequent, several reports have been published regarding vascular complications in children and adults with WBS. For instance ischemic strokes [90, 91], myocardial infarction [100, 101] and even sudden death [102–104]. Despite putative life-threatening complications, it seems that there is no consensus regarding which should be the first line of HTN treatment in WBS [14, 105]. Approximately 30% of patients in our cohort were receiving pharmacological treatment with different type of drugs, including calcium channel blockers, β-blockers, angiotensin II receptor antagonists or others; as has been previously published [105].

Studies in mice harboring a partial deletion that includes the elastin gene demonstrated that the hypertensive phenotype could be reversed genetically by crossing it with a *Ncf1* mutant and pharmacology with losartan (angiotensin II receptor blocker) and apocynin (naturally occurring oxidative stress inhibitor) [17]. Based on the active mechanisms of losartan and that it is a drug approved for HTN treatment in pediatrics, we treated a small cohort of 8 hypertensive patients with WBS with an angiotensin II receptor blocker (losartan) with positive results. Mean, systolic, diastolic and nocturnal blood pressure index were below 1 (corresponding to the 95th percentile) at 12-months after treatment with no adverse effects. Systolic and diastolic nocturnal blood pressure load

also decreased significantly. However, patients still present a relevant number of readings above the 95th percentile. PRA and aldosterone plasma levels were increased at 12-months after treatment. This is unexpected since the blockade of AT1 receptors inhibits the negative feedback loop leading to an increase in renin secretion and increased synthesis of angiotensin I [89]. Oxidative stress markers were also measured and there was a significant decrease in the main lipid peroxidation by-product (MDA). Further studies are needed to corroborate our findings, but these results are evidence that losartan might be a good option for HTN treatment in WBS.

Cardiovascular disease in WBS is caused by the deletion of the elastin gene. However, other factors should be involved to explain the high variability of cardiovascular manifestations in WBS. We have tried in the present study to analyze some of the genetic modifying factors including *NCF1* copy number, variants in the remaining copy of elastin and variants in other genes associated with cardiovascular disease. *NCF1* gene encodes the $p47^{phox}$ subunit of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex. The deletion of a functional copy of *NCF1* has been reported to protect patients against HTN by reducing angiotensin II-mediated oxidative stress [15].

We did not find a significant association with ABPM variables or with the frequency of office HTN. However, when stratified by gender, female patients with only one copy had less frequently HTN. We also studied oxidative stress damage by measuring MDA, which is the principal and most studied product of lipid peroxidation [106]. We found an increased MDA concentration in hypertensive WBS individuals compared to normotensive. Even though, we did not find a significant association patients with two or more *NCF1* copies had increased MDA plasma concentration compared to patients with only one copy. Hypertensive patients had increased MDA concentration compared to normotensive individuals. This supports the hypothesis that patients with HTN also have increased oxidative stress. We did not find a significant association with the remaining oxidative stress parameters measured. Short-term and long-term variability of biomarkers related to oxidative stress are still unclear [107]. Several factors can influence the variability including the time between blood extraction and analysis, diet, lifestyle factors, gender [107, 108].

We found that the variant rs2071307 in *ELN* gene was significantly associated with cardiovascular lesions. Patients that carry the G-allele presented more frequently cardiovascular disease, compared with patients that carry the A-allele. Elastin variants were sequenced in a cohort of 49 patients with WBS and, although an association analysis was not possible due to the population size, the authors identified this variant in some of the patients presenting cardiovascular disease [16]. Further studies are needed that should include a larger cohort to confirm if these or other genetic variants in the remaining *ELN* copy could be a

possible modifying factor of the cardiovascular phenotype.

We also studied 7 SNPs that have been previously associated with hypertension in GWAS or other smaller association studies. Although none of the comparisons remained significant after Bonferroni´s correction, we found interesting associations that warrant further studies to corroborate them. For instance, we found a significant association between *PLCE1* gene and systolic and diastolic office BP index. *PLCE1* encodes a phospholipase enzyme that catalyzes the hydrolysis of the phosphatidylinositol-4,5-biphosphate, which is a signaling pathway involved in the regulation of many physiological processes [109]. *PLCE1* has a relevant role in podocyte development and is a novel loci associated with systolic and diastolic BP [52]. We also found an association between *PLCE1* and cardiovascular alterations. Although *PLCE1* is also expressed in the cardiac myocyte [110], further studies are needed to confirm its implication in cardiovascular disease.

We also found a significant association between *FBLN2* and valvular disease. An association study comparing Down syndrome patients with and without atrioventricular septal defects reported a possible pathogenic variant of *FBLN2* [111]. *FBLN2* encodes an extracellular matrix protein and it down-regulates VEGF, a potent mitogen involved in the regulation of atrioventricular valvuloseptal morphogenesis [112]. Transgenic mouse models have also suggested that VEGF is a specific mediator of heart valve development [112]. Further studies are needed to corroborate if this and other variants in genes involved in heart valve development might be associated with valvular disease in WBS patients.

We have found other association between genes involved in the renin-angiotensin-aldosterone axis and cardiovascular alterations, valvular disease and peripheral pulmonary stenosis. However, at this moment with the information available in the literature we are unable to determine the mechanisms involved for such an association. Further studies are needed to corroborate these results in a larger cohort of WBS patients.

Our results corroborate previous cardiovascular findings in a larger cohort of patients. We have described in great detail ambulatory BP in patients with WBS and have found an increased frequency of nocturnal HTN, and have associated with changes in LVM and geometry. We have treated a small cohort of hypertensive patients with losartan with beneficial results. We have also searched for possible genetic modifiers of cardiovascular disease, finding two novel genes associated with HTN and cardiovascular disease. Due to the high cardiovascular risk of WBS patients and the infrequent but severe complications that can arise, further studies are needed to corroborate our results and to determine the putative long-term implications. This will help to establish better management and pharmacological treatment, diagnose earlier and prevent life-threatening complications.

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SUPPLEMENTARY FIGURES

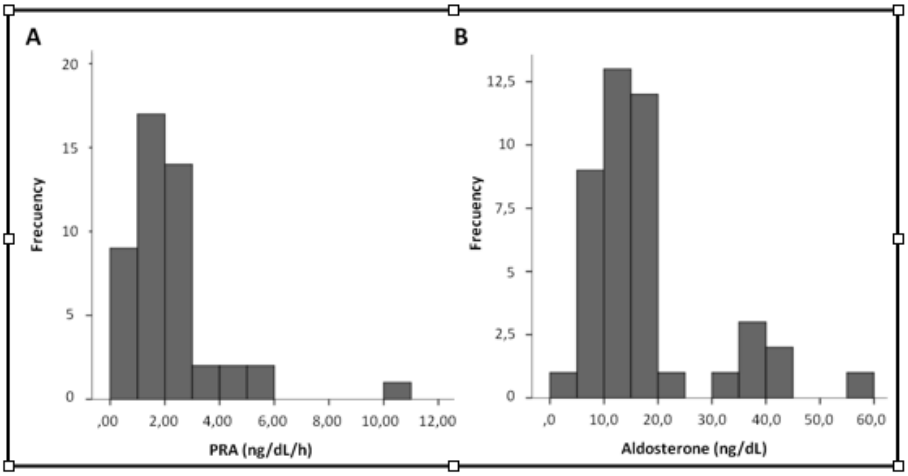

Figure S1. Histogram of: **A.** Plasma Renin Activity (PRA). **B.** Aldosterone plasma levels.

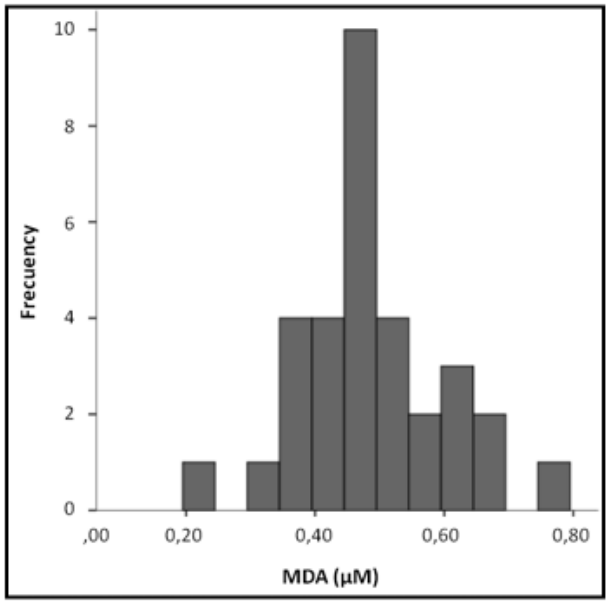

Figure S2. Histogram of malondialdehyde (MDA) plasma values in 32 patients with WBS.

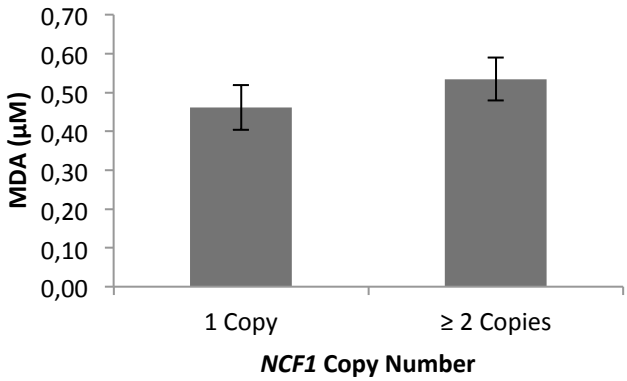

Figure S3. Comparison of MDA plasma concentration with *NCF1* copy number.

SUPPLEMENTARY TABLES

	Adults (≥18 years) n=18	Children (<18 years) n=24
HTN	33% (6/18)	33% (8/24)
SHTN	39% (7/18)	42% (10/24)
DHTN	28% (5/18)	33% (8/24)
Nocturnal HTN	56% (10/18)	58% (14/24)
LVH	30% (3/10)	28% (5/18)
Overweight-Obesity	67% (12/18)	26% (6/23)

Table S1. Ambulatory HTN in pediatric and adult patients with WBS.

	HTN		SHTN		DHTN		Nocturnal HTN	
	No	Yes	No	Yes	No	Yes	No	Yes
Malondialdehyde, (µM)	0,467 ± 0,101	0,534 ± 0,110	0,472 ± 0,111	0,513 ± 0,101	0,462 ± 0,085	0,546 ± 0,131	0,459 ± 0,057	0,519 ± 0,136
Fluorescent Products of Lipid Peroxidation, (µM)	31,71 ± 8,12	30,41 ± 6,06	32,13 ± 8,40	29,99 ± 5,75	31,54 ± 7,82	30,78 ± 6,96	29,61 ± 7,15	32,97 ± 7,58
Advanced Oxidized Protein Products, (µM)	93,83 ± 29,62	119,05 ± 42,27	98,13 ± 37,99	107,8 ± 32,03	93,74 ± 29,62	119,23 ± 42,16	98,50 ± 31,66	105,37 ± 39,82
Protein Carbonyl Group, (nmol/mg protein)	2,91 ± 1,26	3,09 ± 0,97	2,97 ± 1,318	2,95 ± 0,922	3,03 ± 1,37	2,84 ± 0,55	2,81 ± 1,24	3,12 ± 1,098
Oxidized Low Density Lipoprotein, (U/L)	38,08 ± 16,63	46,83 ± 11,22	38,46 ± 17,35	44,62 ± 11,66	39,39 ± 17,09	43,91 ± 11,54	41,26 ± 17,77	40,3 ± 13,34
Glutathione Peroxidase, (U/L)	633,9 ± 76,3	627 ± 126,8	641,8 ± 75,6	615,4 ± 117,2	649,2 ± 86,9	593,2 ± 97,8	635,3 ± 80,2	628 ± 107,1
Glutathione Reductase, (U/L)	68,78 ± 13,01	75,01 ± 4,95	70,08 ± 13,40	71,74 ± 7,64	68,94 ± 13,07	74,64 ± 5,08	67,53 ± 14,55	74,11 ± 5,38

Table S2. Oxidative stress parameters compared with mean, systolic, diastolic and nocturnal hypertension.

	0 Months (Mean ± SD)	12 Months (Mean ± SD)
Dip (%)	9,69 ± 5,736	14,72 ± 4,69
Systolic SD Daytime (mmHg)	12,71 ± 4,538	14,57 ± 2,173
Diastolic SD Daytime (mmHg)	10,76 ± 2,989	12,9 ± 5,94
Systolic SD Nighttime (mmHg)	12,93 ± 5,779	9,86 ± 2,42
Diastolic SD Nighttime (mmHg)	9,02 ± 2,69	8,57 ± 3,88
24h-PP (mmHg)	51,86 ± 8,071	47,73 ± 5,217
Daytime PP (mmHg)	51,86 ± 8,668	47,74 ± 5,525
Nighttime PP (mmHg)	52,14 ± 8,133	48,06 ± 8,352
AASI	0.600 ± 0.196	0.386 ± 0.269

Table S3. ABPM variables at 0 and 12 months after losartan treatment in hypertensive patients with WBS (n=7).

	0 Months	12 Months
Fluorescent Products of Lipid Peroxidation (µM)	30,70 ± 6,42	34,74 ± 4,27
Advanced Oxidation Protein Products (µM)	122,48 ± 43,83	101,91 ± 22,96
Protein Carbonyl Group (nmol/mg protein)	3,23 ± 0,93	3,59 ± 0,78
Oxidized Low Density Lipoprotein (U/L)	46,55 ± 11,96	38,15 ± 15,05
Glutathione Peroxidase (U/L)	608 ± 121,10	457,8 ± 93,53
Glutathione Reductase (U/L)	76,14 ± 3,86	71,44 ± 7,56

Table S4. Oxidative stress parameters at 0 and 12-months after losartan treatment in hypertensive patients with WBS (n=8)

DISCUSSION

Molecular characterization of the 7q11.23 deletion

In this study we have performed a fine molecular characterization of the *de novo* submicroscopic 7q11.23 deletions in a cohort of 720 unrelated patients. We have used several techniques to define the mechanisms participating in the rearrangements, including microsatellite analysis to determine the extension of the deletion and identify the LCR blocks involved, site-specific nucleotide analysis to delimit the breakpoint and PCR amplification and sequencing of junction fragments.

The frequency of atypical deletions in our cohort is 1.6%, including four low-recurrent 2 or 4 Mb deletions extending towards the thelomere, four non-recurrent deletions of 8 and 10 Mb, and one or 1.7Mb. The remaining three smaller deletions have been previously described and characterized [144, 232]. The molecular mechanisms of these deletions could be other than the NAHR, since they have occurred outside the typical SDs and are non-recurrent. The study of the exact breakpoint location of atypical deletions and a detailed clinical evaluation of the patients are of great importance for establishing genotype-phenotype correlations.

Approximately 86% of cases occur due to crossing over events between the centromeric (Bc) and medial (Bm) B-blocks which generate a 1.55 Mb deletion, in agreement with our previous publication [97]. The remaining 12% occur due to crossing over events between the centromeric (Ac) and medial (Ac) A blocks with a deletion size of 1.83 Mb, also in concordance with previous results [97]. The deletion occurs with increased frequency between the B-blocks because of the higher degree of sequence homology between the Bc and Bm blocks (99.6%) compared to the homology between Ac and Am; and because of the shorter distance between the duplicons [97]. In comparison with deletions at 22q11.2 [233], we did not find differences in the parental origin of deletions.

We have further refined the breakpoint location of the 1.55 Mb deletion. Approximately 76% of deletions occur in the proximal half

of the block that contains *GTF2I/GTF2IP1* and *NCF1/NCF1P1* genes. The majority of the deletions occur before the *NCF1* gene (54.2%). This preferential location can be explained because the first half of the block contains a higher percentage of sequence identity (99.8% vs 99.4%) and it also contains five of the seven stretches of continuous identify [97]. Our results corroborate that the crossing over events that generate the 1.55 Mb deletion occur in regions of very high sequence identity [97]. The breakpoint located at *GTF2IRD2* occurs in approximately 24% of cases, generating a chimeric copy composed of the medial block in the first exons and the centromeric block in the last exons. The functional and phenotypical implications of this chimeric copy are still unknown.

The presence of SDs in inverse orientation can predispose to the occurrence of inversions [106], as is the case for the paracentric inversion at 7q11.23. The crossing over between an inverted and a non-inverted chromosome occurs in the last 38 kb absent in the Bc, since crossing over at any other region will most likely result in the generation of an acrocentric or dicentric chromosome that wont segregate correctly during meiosis [97]. The deletion was mediated by a paracentric inversion in the transmitting progenitor in approximately 22% of patients. The presence of a paracentric inversion can predispose to the occurrence of chromosomal rearrangements secondary to chromosomal mispairing during meiosis. This has been observed for other genomic disorders like Sotos syndrome or Angelman syndrome [234, 235]. In the 17q21.31microdeletion syndromes the presence of an inversion is a prerequisite for the generation of rearrangements [138, 236]. In the majority of patients the breakpoint was located at the most distal part of *GTF2IRD2*.

In many recurrent genomic disorders a positional preference for strand exchange has been identified [111, 237, 238]. We have corroborated the previously described 3.4 kb-hotspot located within *GTF2I* for the deletions of 1.55 Mb with a crossover between Bc and Bm in a larger cohort of patients [97]. We have studied 73 cases with deletions in the proximal half of the block, of which 33% amplified

the *de novo* 3.4 kb junction fragment. The recombination rate at this hotspot is approximately 7.6 events per kb. Much higher than the global recombination rate at WBS critical region (1.6 events per kb). In agreement with previous data [97], this hotspot occurs almost exclusively in deletions of paternal origin (17 compared to 1). Previous and unpublished data [97] have shown that the majority of deletions occurring at this hotspot occur due to an intrachromosomal event. Sex-preferential hotspots have also been described in other genomic disorders [111, 239, 240]. In the case of inversion-mediated deletions, we describe two novel hotspots located within *GTF2IRD2* gene, JFINV1 of 1.4 kb and JFINV2 of 2.6 kb. Approximately 15.8% occur in JFINV1 and 38.6% in JFINV2. The recombination rate at these hotspots is 6.4 and 8.5 events per kb, respectively. Also much higher compared to the global recombination rate for inversion-mediated deletions (1.6 events per kb). There was no significant sex-bias observed; however, JFINV1 occurs more frequent in deletions of paternal origin while JFINV2 occurs slightly more frequent in maternal origin deletions. It seems that in WBS there are sex-dependent factors that might influence the position of strand exchange. It is known that the distribution of crossover locations is different between both sexes, females tend to have a lower frequency at the telomeres and higher near the centromeres compared to males [241].

Several *cis*-acting factors can influence the rate of recombination, for instance enrichment of secondary structures, recombination motifs, *Alu* signal recognition motifs, the presence of microsatellites, GC content, higher density of CpG motifs, among others [105, 110, 242]. We observed a higher density of GC content in the last 38 kb of the B block that aligns during meiosis in heterozygous inversion carriers. We also found a higher density of *Alu* repeats and an increase in immunoglobin heavy class switch repeats in all three hotspots. An enrichment of recombination motifs has also been described in other hotspots of recurrent genomic disorders, Charcot-Marie-Tooth type IA and hereditary neuropathy with liability to pressure palsies [238], Sotos syndrome[234] or Smith-Magenis syndrome [112]. *Alu*

sequences can trigger NAHR events leading to different chromosomal rearrangements [243, 244] and its presence can increase the recombination rate by 6% [245].

Trans-acting mechanisms can also influence the rate of recombination. A 13-mer motif was identified in approximately 40% of human hotspots [115] and is a binding site for a highly polymorphic zinc finger protein called *PRDM9* [117]. Sperm donors with non-A alleles had significantly decreased recombination rate [117]. The authors also studied the rate of de novo rearrangements at 17p11.2 and found that non-A alleles were protective against recombination at this locus [117]. Although we found the 13-mer motif in all three hotspots, we did not find differences in the allelic frequencies of A and non-A alleles between transmitters and non-transmitters. Therefore, *PRDM9* effect on recombination rate might be locus specific.

Due to the complex architecture at 7q11.23, other structural variants apart from the paracentric inversion can occur. As previously reported [132], we found a 2.8-fold increase of structural variants (polymorphic block deletions or duplications) in the WBS transmitting group compared to the non-transmitting group. Most of the polymorphic block CNVs were of maternal origin. As in the case of the paracentric inversion, structural variants can also predispose to chromosomal misalignment during meiosis [97, 132].

We have corroborated our findings in an unprecedented large cohort of patients with WBS and contributed with novel findings regarding the mechanisms involved. Further studies are needed to determine which protein or proteins are involved in male-meiosis that could be involved in the generation of a deletion secondary to an intra-chromosomal event. The detailed molecular characterization of the deletion will allow establishing putative genotype-phenotype correlations with the objective of elucidating the clinical relevance of some of the genes involved in the deletion, as well as searching possible pharmacological interventions.

Metabolic abnormalities in Williams-Beuren syndrome

Several metabolic parameters were analyzed in a cohort of 154 patients with WBS, with data available from 69 to 151 cases per parameters; as well as several mouse models with complete and partial deletions of the orthologous WBS locus and searched for causative genes and potential modifiers.

Subclinical hypothyroidism, defined as an elevation of thyroid stimulating hormone (TSH) with normal thyroid hormones, has been reported in 15-30% of patients with WBS [48, 49] and it has been associated with thyroid hypoplasia [48–50]. We found isolated elevated TSH levels in 31.3% of WBS patients with a significant association with younger age. Other authors have also described this association and have postulated two possible explanations [49, 50]. The first explanation is that during the first years of life there is a higher requirement of TSH levels and the hypoplastic gland cannot produce sufficient thyroid hormones resulting in an increase of TSH levels [50]. The other possible explanation is an immaturity of the hypothalamic-pituitary-thyroid axis that resolves with age [49]. At the moment, none of the genes present in the deleted interval have been associated with this phenotype or seem to be involved in thyroid development. However, the metabolic analysis performed in the mouse models, revealed that only the proximal deletion mice had increased TSH levels. This suggests that the haploinsufficiency for one or more genes located at this interval (telomeric from *LIMK1*) could contribute to this developmental phenotype of the thyroid axis.

Alterations in glucose metabolism is also a frequent finding among young adults with WBS [51, 52]. Two different studies in young adults reported a prevalence of glucose intolerance or diabetes mellitus (DM) between 64-75% after an oral glucose tolerance test [51, 52]. One of this studies analyzed β-cell function and insulin response, and attributed that impaired glucose metabolism was associated with a decrease in insulin sensibility in the absence of β-cell dysfunction, autoimmunity or other traditional risk factors of DM [52]. Basal

hyperglycemia was found in 7.4% of our cohort, with only one case of DM. Patients with hyperglycemia had a mean age in the second decade of life which supports the current recommendations of evaluating glucose parameters using oral glucose tolerance test starting from this age [51]. Although we did not find alteration in glucose plasma levels among the studied mice models, the histological analysis revealed morphologic alterations in the pancreatic Langerhans islets in CD mice with a significant reduction in the islets size. This finding might not have a metabolic repercussion at a young age, but may predispose to dysfunction in adulthood. Long-term functional consequences, insulin and glucose levels should be studied in older animals.

Hypotriglyceridemia, defined as triglyceride plasma concentration below the 5th percentile, was found in 18% of individuals with WBS. None of the patients presented extreme values below 2.5 standard deviations. Cholesterol plasma levels in our cohort followed a bimodal curve, with only one patient presenting a value above the 97.5th percentile. This evidence suggests that the lipid profile in patients WBS is characterized by hypotriglyceridemia with low-normal cholesterolemia, which corresponds a priori to low cardiovascular risk profile. This could be a possible compensatory mechanism due to the high cardiovascular risk of WBS patients (including vascular stenosis, HTN, glucose intolerance, obesity). An excellent candidate gene that could be associated with the lipid phenotype is *MLXIPL*. This gene is located in the WBS single-gene copy region and encodes a basic helix-loop-helix zipper transcription factor of the Myc/Max/Mad superfamily [246, 247] that regulates triglyceride synthesis and storage [159]. Several line of evidence supports this hypothesis. First, genome-wide association studies have identified single nucleotide polymorphisms in *MLXIPL* that are associated with triglyceride plasma concentration levels [223, 248, 249]. Second, the murine knock-out model of *MLXIPL* presents decreased lipogenesis, low plasma free fatty acids and decreased adipose tissue [159]. However, we did not find an association between plasma concentration and the previously associated SNP [223] present in the remaining copy of *MLXIPL*. Triglyceride levels were also studied in the mice models at

two time-points. At 4.5 weeks we found a significant reduction of triglyceride plasma levels in the two models with non-overlapping partial deletions (proximal and distal deletion). Unfortunately, the mice model harboring the complete deletion could not be studied at this time due to lack of samples. At 26 weeks, only the DD mice maintained a significant decrease in triglyceride plasma levels. This evidence suggests that the major cause for hypotriglyceridemia observed in WBS patients and the mouse models, is most likely due to the haploinsufficiency of *MLXIPL*. The increased prevalence at a young age could be modified by the deletion of other genes in the interval, as well as environmental factors such as diet.

Mildly elevated levels of total bilirubin were observed in approximately 20% of patients with WBS. The profile of bilirubin levels (mostly unconjugated) and the absence of hemolysis or hepatic disease indicated that the most probable cause was Gilbert syndrome (OMIM 143500). Gilbert syndrome is characterized by mild intermittent jaundice with an estimated prevalence of 6-8% [250, 251]. Gilbert syndrome is mainly caused by a hypomorphic allele due to a polymorphism in the promoter region of *UGT1A1* gene [250]. *UGT1A1* encodes the enzyme glucoronosyltransferase responsible for the glucoronidation essential for bilirubin excretion [250, 252]. The penetrance of the hypomorphic allele is incomplete, approximately 40% depending on the criteria used to define the phenotype. Bilirubin levels were significantly associated with the *UGT1AI* genotype in our cohort. We found an increased frequency of unconjugated hyperbilirubinemia or Gilbert syndrome in WBS, with a penetrance of approximately 73% for the hypomorphic allele (3-fold increase compared to the general population). However, *UGT1A1* gene might not be the only cause of this phenotype. Approximately 13% of patients homozygous for the fully functional allele also presented hyperbilirubinemia and all the mice models studied had normal bilirubin plasma levels and normal gene expression. We also found a significant correlation between bilirubin z-score values and triglyceride z-score values, suggesting a common pathogenic mechanism for the lipid and bilirubin alterations.

Other relatively frequent alterations in our cohort were increased total protein and albumin levels, and iron metabolism (increased transferrin and iron levels). Further studies are needed to corroborate these findings, determine the physiological cause and the clinical implications of these alterations.

In this study we have described several unreported metabolic alterations with relative frequency in children and adults with WBS. We have tried to identify putative candidate genes by gene association analysis and using complete and partial deletion mouse models. These results should be studied in greater detail to better define clinical guidelines and prevent long term complications. Awareness of the metabolic alterations in WBS will prevent unnecessary studies and interventions, as well as to identify the changes that should be monitored to avoid potential complications.

Cardiovascular manifestations and evaluation in Williams-Beuren syndrome

Cardiovascular manifestations are present in approximately in 75% of patients with WBS and are caused by the hemizygous deletion of the elastin gene [12]. The main hallmark of the cardiovascular disease observed in WBS patients is the stenosis of medium and large-sized arteries, the most frequent being SVAS and PPS, although any artery can be affected [12]. The prevalence of arterial hypertension in WBS patients is highly variable [12, 33]. In this study we have analyzed information about cardiovascular disease, including HTN, in a cohort of 136 patients with WBS. We have also analyzed the results of echocardiography, abdominal Doppler ultrasound and ABPM in a smaller cohort of patients. We have tried to search for possible genetic modifiers of the cardiovascular phenotype, including *NCF1* copy number, genetic variants in the remaining copy of elastin and SNPs previously associated with HTN or cardiovascular disease. Finally, hypertensive patients diagnosed with ABPM were treated with an angiotensin II receptor blocker and followed-up at 12 months to determine its efficacy.

Approximately 76% of patients in our cohort presented cardiovascular disease, including SVAS, PPS, mitral valve prolapse (MVP), coarctation of the aorta, valvular disease and interventricular or interatrial septal defects, with similar frequencies to what has been reported previously in the literature [12]. MVP and aortic and mitral insufficiencies are the most common valvular diseases. The mitral valve is composed of three layers, the closest one to the atrium is enriched with elastic proteins that mitigate leaflet stretch during systole [253]. Therefore, the deficit and abnormal production of elastin in WBS patients could explain this abnormality.

Cardiovascular disease occurred significantly more frequent in male patients and with increased severity, as has been described in a previous study [30]. The authors postulated that the difference observed could be secondary to sex hormones, which are known to be important regulators of cardiovascular development and function [30]. We also found that patients presenting PPS and SVAS were significantly younger. This is also in agreement with previous results [31], PPS tends to improve with age likely due to changes in the arterial mean tension [29].

Increased medial thickness was observed in approximately 14% of WBS patients. This has also been previously described and it demonstrates the extensiveness of vascular alterations in WBS patients [254]. Stenosis of abdominal arteries was found in 43% detected by Doppler ultrasound, the arteries involved were the descending aorta, celiac trunk, superior mesenteric artery and the renal artery. In a cohort of 14 individuals a CT angiogram was also performed. Arterial stenoses were corroborated by the CT angiogram, in some cases detecting new alterations. However, in 7 of the 9 patients with a normal Doppler ultrasound, the CT angiogram reported stenosis of at least one abdominal artery. A previous study reported abnormalities in the abdominal aorta in 80% of patients using aortography [255]. Approximately 57% of patients of our cohort had middle aortic syndrome using CT angiogram. Middle aortic syndrome is characterized by narrowing of the descending thoracic or abdominal

aorta, with stenosis of the renal and other visceral arteries [28], its frequency in WBS is uncertain with a range between 2-70% [29]. CT angiogram has a greater detection rate and precision (greater number of visualized abdominal branch stenoses) over Doppler ultrasound and justifies the choice for diagnosis and follow-up despite of the radiation risks. Due to the high incidence of system arterial stenosis, patients with WBS should be evaluated to detect promptly and control if complications, such as renosvascular HTN and portal HTN, arise [12, 256].

HTN is also a frequent manifestation in WBS patients. However, the underlying mechanism is still unknown. It has been observed that the deficit in elastin causes morphologic alterations in the arterial wall leading to luminal narrowing, arterial stiffness and decreased vascular distensibility which could explain the HTN in WBS patients [12, 18]. Office HTN was found in 40% of patients with WBS, with a higher frequency of systolic compared to diastolic HTN. Ambulatory HTN was found in 33% of patients; with a higher frequency of systolic HTN compared to diastolic HTN. Systolic HTN in adults is associated with increased arterial stiffness, while in children it has been thought to be secondary to sympathetic nervous system hyperactivity [257]. It is possible that in WBS patients systolic HTN is associated to an increased cardiac afterload secondary to arterial stiffness. HTN in WBS should be considered as a manifestation of a generalized arteriopathy rather than secondary to renal hypoperfusion [26].

Surprisingly, we found that 57% had nocturnal HTN and in 29% it was isolated (with a normal mean blood pressure). Isolated nocturnal HTN is found in 7% of the general population [258]. Previous studies using ABPM in patients with WBS did not study this variable [33–35] but analyzed circadian variation and found a blunted nocturnal decrease in normotensive individuals [34] and in 47% normo or hypertensive patients [35]. Blunted nocturnal BP can be caused by multiple factors, including increased synaptic activity, poor quality of sleep, obesity, diabetes, among others [38, 259]. A recent study in WBS patients using ABPM found an increased nocturnal HR, with an

impaired physiological reduction in day-night shift [39]. The authors also postulate that these alterations could be secondary to sympathetic hyperactivity [39]. On the other hand, several studies have described sleep disturbances in children and adults with WBS [260, 261]. Further studies are warranted to corroborate our results and search for its etiology, since nocturnal HTN and blunted nocturnal response are two independent variables associated with end-organ damage and higher cardiovascular morbility [36, 37].

We found no significant difference in age between normotensives and hypertensives. There was also no significant difference between the frequency of mean, systolic, diastolic and nocturnal HTN between adults and children. The rate of LVH was also the same between both groups. Although HTN has been thought to be more frequent in adults [12, 33], this demonstrates that it can occur at any age. Given that complications secondary to HTN have also been described in childhood [41, 262–266], it should be recommended to measure blood pressure (BP) in children.

Left ventricular hypertrophy (LVH) was found in approximately 26% of our cohort. A previous study reported LVH in 56% of WBS patients; however, they cohort was smaller [40]. LVH was significantly associated with mean, systolic, diastolic and nocturnal HTN. This is evidence that untreated HTN in WBS patients can have a clinical impact since LVH is a sensible marker of target organ damage [267]. Approximately 60% of patients had an abnormal left ventricular geometry, which is also similar to a previous study in WBS patients [40]. All hypertensive patients presented an abnormal left ventricular geometry, as well as normotensive patients with other cardiovascular lesions (SVAS, PPS, MVP, coarctation of aorta). There was only one case that did not present HTN or cardiovascular lesions and had an abnormal left ventricular geometry. This is also in agreement with a previous study that concluded that mild left ventricular functional and textural abnormalities may be even detected in absence of significant SVAS or HTN [40]. Left ventricular geometry may add prognostic information in the assessment of hypertensive individuals [268].

As in the general population and previous studies in WBS patients, we found a significant association between blood pressure indexes and LVH with body mass index (BMI) [34, 269]. It should be highly recommended to WBS patients to maintain a BMI within the normal limits due to their high cardiovascular risk secondary to HTN, glucose intolerance and vascular stenosis.

Although vascular complications in WBS patients are infrequent, they tend to be life threatening and can occur irrespective of the age. For instance ischemic strokes, myocardial infarction or even sudden death [41, 262–266]. However, despite this complications there seems not be a consensus about which should be the first line of treatment for HTN in WBS. A previous study also mentioned that there is no data available to recommend specific drug for patients with WBS [12]. We treated a cohort of 8 hypertensive patients with losartan (angiotensin II receptor blocker), since a previous study postulated that the pathogenic mechanism of HTN was that arterial stiffness leaded to a chronic activation of the NOX system by angiotensin II [31]. We found a significant decrease in mean, systolic, diastolic and nocturnal blood pressure index at 12-months after treatment without significant adverse effects. Oxidative stress biomarker was also significantly decreased after 12 months of treatment. This study should be replicated in a bigger cohort, but these results are evidence that losartan might be a good option for HTN treatment in WBS.

One of the putative genetic modifying factors of cardiovascular disease in WBS patients is *NCF1* copy number [31]. *NCF1* encodes for the $p47^{phox}$ subunit of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex and is located in the segmental duplications that flank the region and mediate most of the deletions [97]. As it was mentioned in the molecular mechanisms chapter, the functional copy of *NCF1* is located in the medial block, while the other two blocks (centromeric and telomeric) each contain a pseudogene [270]. Depending on the breakpoint location of the deletion, some patients can lose a functional copy [97]. However, secondary to gene conversion events some patients can also gain

functional copies [97, 121]. The deletion of a functional copy of *NCF1* has been reported to protect patients against hypertension by reducing angiotensin II-mediated oxidative stress [31]. We found a significant association with office HTN when stratified by gender, female patients with only one copy presented less frequently HTN compared to female patients with two or more functional copies. Although not significant, hypertensive patients had higher oxidative stress compared to normotensive patients.

A significant association was found between *PLCE1* and systolic and diastolic office blood pressure index. *PLCE1* encodes for phospholipase enzyme, plays a relevant role in podocyte development and has recently been associated with systolic and diastolic blood pressure [272, 273]. We also found an association between this SNP and cardiovascular disease and valvular disease. *PLCE1* is also expressed in cardiac myocytes. Further studies are needed to corroborate our results.

We found a significant association between a non-synonymous SNP in the remaining copy of elastin and cardiovascular alterations. This SNP has been previously identified in patients of WBS [271], but no association was found due to their small cohort number. Further studies are needed to corroborate whether this variant alters the amount of elastin protein and is associated with an increased frequency of cardiovascular alterations.

A SNP in *FBLN2* was associated with valvular disease. *FBLN2* variants has also been associated with atrioventricular septal defects in patients with Down syndrome [274]. *FBLN2* encodes for an extracellular matrix protein and it down-regulates VEGF, a potent mitogen that has been suggested to be a specific mediator of heart valve development [275].

In this study we corroborate previous findings with respect to cardiovascular disease and office HTN. We also describe that stenosis of abdominal arteries is very frequent in WBS patients and that CT angiogram might be a better imaging test to detect them. We have

described in greater detail ambulatory HTN, identifying a high percentage of patients with nocturnal HTN or with an abnormal blunted nocturnal decrease that should be corroborated in other cohorts due to its clinical implications. We describe that untreated HTN has functional consequences on left ventricle morphology that also are associated with increased cardiovascular morbidity. We also demonstrate that losartan could be potential drug for HTN treatment in WBS patients. We have searched for putative candidate genes associated with this phenotype, describing two novel genes (*PLCE1* and *FBLN2*) possibly associated.

Concluding remarks

WBS is a genomic disorder, caused by a deletion at chromosomal band 7q11.23. As in other genomic disorders, the genotype – phenotype correlations are complicated because of the gene-gene interaction and because more than one gene can be involved in a given phenotype. Several approaches have been used to elucidate on the function of the genes involved in the deletion, including the study of atypical deletions, single gene and partial or complete deletion mouse models and, finally functional assays. Until know the only gene that we know its clinical implication with certainty is elastin with the cardiovascular phenotype. There are evidence pointing to the implication of some of the remaining genes with other phenotypes, but further studies are needed to corroborate those results.

One of the previous most important steps that are needed to fully be able to study gene – phenotype correlations is a detailed molecular characterization of the deletion. The objectives of this study are to increase the knowledge of the mechanisms involved in non-allelic homologous recombination, search for strand preference locations of the breakpoints and search for putative *cis* and *trans*-acting factors that could predispose the rearrangements. However, indirect objectives of a detailed molecular characterization is that the results obtained will allow us to correlate with specific phenotypic characteristics and determine whether the size of the deletion, the parental origin or other

variable deleted genes (*NCF1* or *GTF2IRD2*) could be associated with specific phenotypes.

The importance of establishing genotype-phenotype correlations lies not only in increasing the knowledge of the clinical characteristics and gene function, but it will also allow us to search for possible therapeutic interventions. For instance, after *NCF1* was described as a putative modifying factor for HTN [31] studies were performed in mice harboring a partial deletion that includes elastin gene and that presented HTN [177]. The hypertensive phenotype of the mice was reverted with the use of losartan (angiotensin II receptor antagonist) or apocynin (a natural antioxidant)[177]. We have treated a small cohort of hypertensive patients with losartan with positive results. This is an important first step on defining the drug that should be used to treat HTN in WBS patients.

The results of this thesis project describe the molecular mechanisms involved in the generation of WBS deletion, an unreported metabolic phenotype and a detailed description of the cardiovascular phenotype of WBS. This thesis project also illustrates the interaction between a detailed molecular characterization and a complete phenotypical analysis in the quest of elucidating putative genotype-phenotype correlations, along with the use of gene-association analysis and animal models

CONCLUSIONS

CONCLUSIONS

We have performed a fine molecular characterization of the recurrent 7q11.23 deletion in 720 patients with WBS. The great majority of deletions had occurred through crossing-over events between misaligned centromeric and medial B-blocks. Approximately 30% of deletions are facilitated by structural genomic variants in the transmitting progenitor, either a 2 Mb paracentric inversion (22%) or large (>100 Kb) copy number variations in the segmental duplications (5%). We have further defined and narrowed down to a 1.4 kb region with intron 19 of *GTF2I*, a previously identified hotspot for NAHR in WBS deletions. This hotspot is almost exclusive of deletions of paternal origin and mediated by intrachromosomal exchanges in meiosis. We have also identified two novel hotspots for inversion-mediated deletions at *GTF2IRD2*, with show also a slight gender bias and always occur through interchromosomal exchange. Thus, sex-dependent factors might influence the preferential position of strand and chromatid exchange resulting in the WBS deletions. Putative cis-acting factors that could facilitate the recombination at these three hotspots include higher GC content and the presence of repetitive elements. *PRDM9* does not seem to be associated with the generation of a deletion in WBS, since higher functional variants at this gene were not more prevalent in the transmitting parents with offspring with WBS.

We have analyzed the metabolic phenotype in a cohort of WBS patients. We describe previously reported metabolic alterations including subclinical hypothyroidism and hyperglycemia. We describe novel metabolic alterations, including a lipid profile characterized by hypotriglyceridemia with low-normal cholesterolemia and increased total bilirubin, mostly unconjugated, in a relevant proportion of individuals. Hyperbilirubinemia correlated with hypothyroidism and hypotriglyceridemia suggesting common pathogenic mechanisms. Haploinsufficiency at MLXIPL, a gene present in the WBS critical region, and increased penetrance for hypomorphic alleles at the *UGT1A1* promoter, main cause of unconjugated hyperbilirubinemia,

might explain the lipid and bilirubin alterations, respectively. We also report alterations in total protein and albumin plasma levels, as well as alterations in iron metabolism. The early diagnosis, management and follow-up of these metabolic alterations can help prevent potential complications and avoid unnecessary tests and interventions.

We have analyzed cardiovascular manifestations including HTN in a cohort of WBS patients. Cardiovascular disease was present in 75% of patients, SVAS and PPS being the two most frequent pathologies. We also report stenosis of abdominal arteries in a relevant number of individuals. CT angiogram seems to be a better diagnostic technique for these alterations compared to abdominal Doppler ultrasound. We analyzed ABPM data in a smaller cohort of patients reporting ambulatory HTN in 33% of patients. However, we also describe nocturnal hypertension (sometimes isolated) and blunted nocturnal decrease in a relevant number of patients. Structural alterations were detected by echocardiography, including LVH and an abnormal LV geometry. Both conditions associated with HTN. BMI was also found associated with HTN and LVH. *NCF1* copy number was associated with office HTN in female patients. A SNP in *PLCE1*, previously associated with systolic and diastolic blood pressure, was significantly associated with HTN and cardiovascular disease. Finally, a variant at *FBLN2*, a down-regulator of a potent mitogen involved in heart valve development, was associated with valvular disease in WBS patients.

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LIST OF ACRONYMS

ABPM	Ambulatory Blood Pressure Monitoring
aCGH	Array Comparative Genomic Hybridization
ADHD	Attention Deficit Hyperactivity Disorder
BMI	Body Mass Index
CD	Complete Deletion
CNV	Copy Number Variants
CSF	Cerebrospinal Fluid
CV	Cardiovascular
DNA	Deoxyribonucleic acid
DD	Distal Deletion
DM	Diabetes Mellitus
HTN	Hypertension
LCR	Low Copy Repeats
LV	Left Ventricle
LVH	Left Ventricular Hypertrophy
MAS	Middle Aortic Syndrome
MIP	Multiple Inversion Probe
MPS	Massive Parallel Sequencing
MVP	Mitral Valve Prolapse
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAHR	Non-allelic Homologous Recombination
NGS	Next Generation Sequencing
PCR	Polymerase Chain Reaction
PD	Proximal Deletion
PPS	Peripheral Pulmonic Stenosis
PSV	Paralogous Sequence Variants
SD	Segmental Duplications
SSN	Site-specific Nucleotide
SVAS	Supravalvular Aortic Stenosis
TSH	Thyroid Stimulating Hormone
US	Ultrasound
WBS	Williams-Beuren syndrome

ANNEX

Aspectos Clínicos y Neuroconductuales del Síndrome de Williams-Beuren

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Revista Asociación Síndrome Williams España N°10, 2011.

El Síndrome de Williams-Beuren (SWB) es un trastorno del desarrollo que afecta a múltiples sistemas, incluyendo problemas neurocognitivos con un perfil característico y diversos problemas médicos. Está causado por una pérdida de material genético en el cromosoma 7, en la banda cromosómica 7q11.23. En la mayoría de casos la pérdida de material involucra entre 26 y 28 genes. Sin embargo, un pequeño número de individuos con cuadros clínicos parciales (1%) presentan una deleción atípica menor del intervalo. El estudio clínico y molecular de estos casos ha permitido definir la contribución individual de varios de los genes a las características clínicas y neuroconductuales de los pacientes con SWB, tanto las manifestaciones en el sistema cardiovascular y el tejido conectivo, como las características craneofaciales, y problemas cognitivos [1,2]. Los últimos datos reafirman que la pérdida del gen *GTF2I* acompañado o no de la pérdida de otro gen de función relacionada, *GTF2IRD1,* es la principal causa del perfil neurocognitivo y de algunos rasgos craneo-faciales. Estos genes codifican factores reguladores de la transcripción, actuando por tanto sobre otros genes del genoma humano.

También se ha estudiado recientemente los efectos que la pérdida de una copia de los 26-28 genes críticos tiene sobre la función global de la célula, analizando los patrones de expresión de genes de todo el genoma en células de sangre o piel de personas con SWB [3]. Los datos sugieren que algunos genes delecionados junto con otros afectados de manera secundaria se pueden agrupar en vías de funcionamiento comunes que explican, al menos en parte, el mecanismo de la disfunción responsable de alguna de las alteraciones que aparecen en el SWB. Por ejemplo, parece que hay varios genes implicados en el mantenimiento de la estructura del citoesqueleto de la célula nerviosa tanto en la retina como en diversas neuronas, como se observa en la figura 1. Esta alteración morfológica y funcional de las neuronas de diversas regiones cerebrales y retina puede ser responsable de los

problemas neurocognitivos y de construcción visuo-espacial.

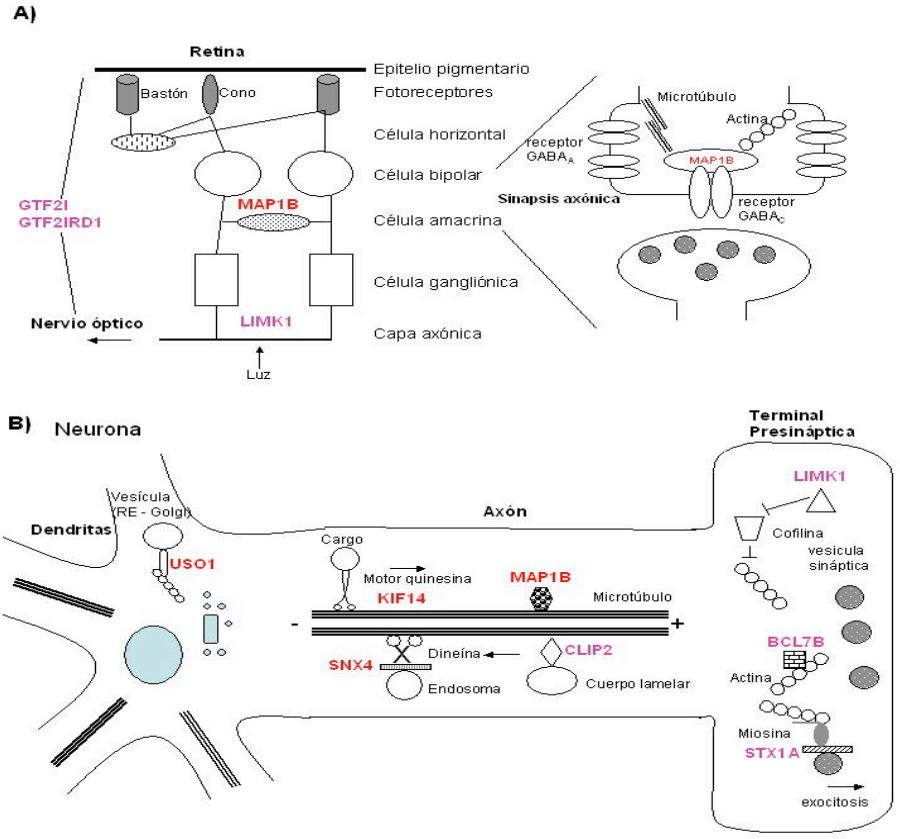

Figura 1: esquema de las vías de señal posiblemente afectadas en el SWB, en células de la retina (A) o una célula neuronal con el cuerpo celular el axón y varias dendritas (B). La posición donde actúan los productos de los genes afectados en el SWB está indicada con el símbolo de cada gen. En morado están los genes relevantes de la deleción típica, mientras en rojo se representan otros genes afectados de manera secundaria.

La enorme contribución a la investigación en el SWB por parte de numerosos grupos alrededor del mundo en los últimos años ha llevado a que recientemente se haya publicado una extensa actualización de los avances en este campo en una revista profesional de gran prestigio como el New England Journal of Medicine [1], y que otra revista de gran difusión entre profesionales de la genética, American Journal of Medical Genetics, haya dedicado una edición especial monográfica más detallada al SWB, enfatizando los avances en el conocimiento de sus causas y mecanismos, sus manifestaciones clínicas,

especialmente el perfil neurocognitivo, y cuestiones relativas al asesoramiento genético de las personas con SWB y sus familias. En los siguientes párrafos resumiremos algunos de estos aspectos, así como las intervenciones que se pueden realizar en cada uno de ellos.

1. Intolerancia a la glucosa

Existen datos relevantes del estudio del metabolismo de la glucosa en adultos con SWB (mayores de 20 años) [4]. Cuando se les administra una prueba de tolerancia oral a la glucosa, el 75% presentan una curva de glucosa anormal, cumpliendo con criterios diagnósticos bien para diabetes o para intolerancia a la glucosa. Los niveles de hemoglobina glicosilada se encuentran no obstante dentro del rango de la normalidad. El metabolismo de la glucosa se encuentra alterado, tanto en los casos con un índice de masa corporal por debajo de 25 (60%), como el los que lo tienen por encima de 25 (80%). Por tanto, el sobrepeso u obesidad no son la única causa de esta disfunción metabólica, aunque pueden ser factores agravantes. Estos datos demuestran una prevalencia muy alta de intolerancia a la glucosa en adultos con SWB, y sugieren que se debe monitorizar el posible desarrollo de diabetes mellitus, realizando ocasionalmente pruebas de tolerancia oral a la glucosa. Además es importante prescribir de manera preventiva un estilo de vida activo, con programas de ejercicio regular y evitación de dietas hipercalóricas. Cuando la alteración es evidente, se precisará un tratamiento farmacológico específico. Los estudios genéticos también han contribuido a determinar cuáles son las vías del metabolismo de la glucosa que funcionan mal en las células de personas con SWB, especialmente la glicólisis y neoglucogénesis (Fig 2) [3].

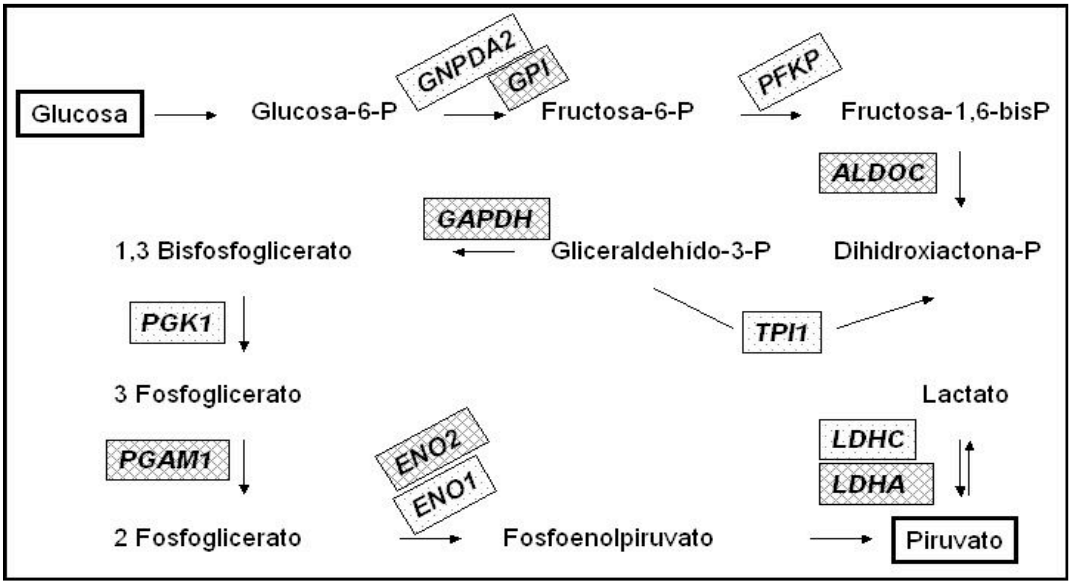

Figura 2: esquema del metabolismo de la glucosa con los diversos genes cuya expresión está alterada en personas con SWB mostrados en recuadros.

2. Función auditiva

El SWB se asocia con frecuencia a patología auditiva. Los niños con SWB presentan frecuentemente otitis media crónica en edad temprana y hay una pérdida auditiva neurosensorial evidente en 63% de los escolares con SWB, comparado con sólo un 7% de la población general [5]. En los adultos, el 92% presentan una pérdida auditiva de leve a moderada-severa, siendo en el 50% de los casos neurosensorial. Estos datos indican que la pérdida auditiva en personas con SWB es lentamente progresiva.

Parece claramente conveniente realizar una evaluación auditiva periódica en las personas con SWB, incluyendo una timpanometría de multi-frecuencia. También es aconsejable que los niños con SWB se sienten en el centro de la primera fila de clase. Para prevenir la progresión de la afectación auditiva, asumiendo una posible predisposición al daño inducido por el ruido, se recomienda evitar o moderar la exposición a ruidos sostenidos como a la aspiradora, los fuegos artificiales, los acontecimientos públicos en lugares cerrados, e incluso el uso de dispositivos personales de música.

3. Características cognitivas y de conducta

Las personas con SWB tienen un fenotipo neuroconductual fácilmente reconocible, con

afectación de la capacidad intelectual, lenguaje, comportamiento adaptativo y personalidad [6,7].

Es conveniente evaluar la capacidad intelectual en cada individuo con SWB con el objetivo de identificar las fortalezas y debilidades, y así poder implementar intervenciones en áreas concretas. Las fortalezas relativas generalmente observadas en el SWB son las áreas de lenguaje, el razonamiento no verbal y la memoria verbal de corta duración; mientras que hay una debilidad evidente en la construcción visuoespacial. La mayoría presentan una discapacidad intelectual leve a moderada [6,7]. La evaluación que se utiliza de manera estándar para determinar el cociente intelectual (CI) es el test de inteligencia de Weschler (con adaptaciones según edad), que consta de pruebas que valoran la habilidad de razonamiento no verbal y también la habilidad espacial.

Con relación al lenguaje, los niños con SWB presentan generalmente un retraso en su adquisición y en la producción de palabras. El vocabulario receptivo, que se puede evaluar con el Test de Peabody (vocabulario en imágenes), ha sido identificado de manera consistente como una de las fortalezas relativas. El lenguaje expresivo también es considerado como un área relativamente preservada. No obstante, hay una importante variabilidad y la mayoría de las personas con SWB tienen una gran dificultad con el vocabulario conceptual, que incluye términos relacionados con el espacio, tiempo, cantidad y dimensiones. También presentan dificultades con la pragmática, la manera en que el contexto influye en la interpretación del significado.

El comportamiento adaptativo se refiere a las habilidades conceptuales, sociales y prácticas necesarias para el buen funcionamiento del día a día. Se suele evaluar mediante la escala de madurez social de Vineland, basada en una encuesta contestada por los padres. Las personas con SWB obtienen resultados mejores en la sociabilización y comunicación, mientras que funcionan peor en las prácticas de la vida diaria y habilidades motoras. El comportamiento adaptativo es quizá una de las áreas en la que los cuidadores pueden tener un impacto mayor sobre el desarrollo de habilidades del niño.

La personalidad de las personas con SWB ha sido descrita como empática, hipersociable y desinhibida socialmente. Sin embargo, a pesar de su gran gregarismo, estos niños suelen tener dificultades para hacer y mantener amistades, más marcadas en edad adulta.

Los programas de intervención deberán estar enfocados a las distintas áreas anteriormente mencionadas. El entrenamiento en habilidades sociales pretende mejorar las habilidades de conversación (la distancia física apropiada, cómo y cuándo interrumpir, etc..), las habilidades de juego (jugar con otros, compartir), el manejo de amistades (llamar la atención de manera positiva) y las

habilidades para manejar la emoción (reconocer los sentimientos, resolver el problema). La terapia de lenguaje es muy importante desde edades tempranas, así como en edad preescolar y durante los años escolares. La terapia debería estar enfocada a todos los aspectos del lenguaje y la comunicación, con un abordaje multidisciplinar involucrando a terapeutas, profesores, educadores especiales y padres del niño. En cuanto a la lectura, parece que los niños que aprenden a leer mediante métodos fonéticos (basados en sonido) pueden tener mejores resultados que los que han aprenden mediante la enseñanza integral (vocabulario y gramática).

4. Ansiedad y psicopatología en niños y adolescentes

Los problemas psicopatológicos que se presentan con mayor frecuencia en personas con SWB son el Trastorno por Déficit de Atención e Hiperactividad y el Trastorno de Ansiedad [7]. En un estudio longitudinal en niños y adolescentes con SWB (entre 4 y 14 años), el 82% de ellos recibió el diagnóstico de trastorno de ansiedad en algún momento del estudio y el 62% presentó ansiedad crónica persistente [8]. Los trastornos de ansiedad más frecuentes fueron fobias específicas, pero también el trastorno de ansiedad generalizado. El 29% fueron tratados con fármacos en algún momento del estudio, sin que fuera efectivo en más de dos terceras partes de los casos. Un 18% recibieron psicoterapia, pero el 75% de los tratados continuó cumpliendo los criterios para el diagnóstico de ansiedad crónica.

Estos datos documentan que existe una alta prevalencia del trastorno de ansiedad, en personas con SWB, que no remite y tiende a generalizarse con el tiempo. A pesar de ello, existe todavía poca investigación en este aspecto concreto y no hay pautas claras de actuación ni tratamiento eficaz. Es importante desarrollar investigación más dirigida en este sentido, al objeto de establecer programas de prevención e intervención adecuados.

5. Asesoramiento genético en adultos

El SWB ocurre casi siempre de manera esporádica, por un error genético que se produce de nuevo. Sin embargo, las personas con SWB pueden transmitir la alteración genética y todas sus manifestaciones al 50% de sus hijos, y existen algunos casos conocidos con este tipo de transmisión autosómica dominante.

Se ha realizado un estudio muy interesante valorando la receptividad al asesoramiento genético en 43 adultos con SWB. El objetivo era determinar si las personas adultas con SWB podrían adquirir el conocimiento básico para explorar las variables que influyen en la toma de decisiones sobre tener descendencia, y además conocer su nivel de satisfacción en relación con el asesoramiento brindado [9].

Se utilizaron dos métodos previamente descritos para el asesoramiento específico a personas

con discapacidad intelectual: 1) transmitir datos básicos utilizando un vocabulario simple y repitiendo la información cuando era necesario, utilizando preguntas simples y cerradas (con respuesta sí o no), en vez de utilizar preguntas abiertas; 2) abordaje del tipo psicosocial, con un mayor enfoque en los sentimientos y actitudes del paciente. El procedimiento utilizado fue una consulta inicial en la que se averiguó el nivel de conocimiento previo de los participantes y se proporcionó a continuación información genética básica sobre el cuadro clínico y riesgo de transmisión a la descendencia. Se realizó un cuestionario ulterior en visita post-asesoramiento para valorar el nivel de aprendizaje y entendimiento adquirido y mantenido, así como el grado de satisfacción y si recomendarían el asesoramiento para otras personas con SWB.

Con respecto al primer objetivo, los individuos con SWB aprendieron algunos conceptos básicos tras la sesión de asesoramiento. El 81% respondió correctamente a la pregunta sobre el riesgo de transmitir la enfermedad a su descendencia y el 64% pudieron mencionar algún dato adicional que aprendieron durante la primera visita de asesoramiento. Con relación al segundo objetivo del estudio, el factor que podría influenciar más la decisión de tener descendencia fue la cuestión sobre cómo se sentirían si su hijo tuviera SWB. El 49% respondió que se sentiría bien o feliz, el 44% se sentiría triste y el 5% estaba inseguro. Un tema recurrente dentro de los individuos que se sentirían tristes, era el estigma facial que ellos a su vez lo habían sentido. El cualquier caso, el 98% de los participantes estaban satisfechos con la atención y recomendarían que se diera información y asesoramiento genético a otras personas con SWB.

Este estudio ha demostrado que el asesoramiento genético directamente a personas adultas con SWB puede ser útil, si bien es recomendable limitar la cantidad de información que se aporta en la sesión y utilizar ayudas gráficas, así como quizá repetir en el tiempo para conseguir la reafirmación.

7. Conclusiones

Las revisiones y publicaciones recientes que se han resumido en este manuscrito demuestran los avances significativos que han sido logrados en los últimos años en la comprensión de los aspectos genéticos, clínicos y neuroconductuales del SWB. Existen ya guías clínicas establecidas, así como pautas de conducta y tratamientos recomendados para diversos aspectos del cuadro clínico. Además, se han generado otros recursos importantes incluyendo modelos animales que van a seguir aportando información relevante en los próximos años. Sin embargo, todavía es necesario profundizar más con investigación clínica y básica, para definir mejor la función de los genes y vías involucradas en las diversas características clínicas,

así como los factores modificadores responsables de la variabilidad. Ello permitirá optimizar las recomendaciones de seguimiento e intervención que se pueden emplear en la práctica, y disponer de nuevas estrategias terapéuticas, tanto farmacológicas o de terapias avanzadas como conductuales.

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La Transición del paciente con síndrome de Williams Beuren: Manejo del Paciente Adulto

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El síndrome de Williams o Williams-Beuren es un trastorno del neurodesarrollo causado por una pérdida de material genético (deleción heterocigota) en la región 7q11.23 del cromosoma 7. Cursa con una afectación multisistémica que incluye rasgos faciales típicos, problemas cardiovasculares (estenosis de arterias medianas y grandes e hipertensión arterial), `posibles alteraciones endocrinas (talla baja, intolerancia a la glucosa, hipercalcemia transitoria) y del tejido conectivo (escoliosis, hernias, divertículos), y un perfil cognitivo caracterizado por debilidades visuoespaciales e hipersociabilidad.

Debido a la afectación de múltiples órganos y sistemas, las personas con síndrome de Williams deberían realizar un seguimiento periódico en consultas multidisciplinares compuestas por diversos especialistas como genetistas, cardiólogos, ortopedas, nefrólogos y psicólogos, entre otros. Si bien durante la infancia y adolescencia la coordinación del manejo y seguimiento de por los diversos especialistas para cada niño suele estar bien establecida, por el pediatra o las unidades de genética, en la mayoría de los casos no existe una buena transición del cuidado pediátrico al cuidado del adulto. El cuidado del paciente adulto suele ser menos organizado y coordinado, en parte por falta de conocimiento y experiencia por parte de los especialistas que deberían implicarse. Se ha demostrado que el uso de guías clínicas o protocolos de seguimiento es fundamental para una adecuada transición (Schrander-Stumpel, 2007).

Tras visitar consecutivamente en el año 2012 a 22 adultos con síndrome de Williams que no estaban en seguimiento regular por nuestro servicio, se constató que el 23% (5/22) no realizaban seguimiento periódico médico por ningún especialista y el 50% (11/22) no realizaba seguimiento cardiológico. En algunos casos, los servicios de cardiología habían dado el alta al paciente debido a que se encontraba asintomático.

Al igual que se ha publicado mediante el estudio de personas adultas con síndrome de Williams en otros países (fundamentalmente Reino Unido, Alemania y Estados Unidos) (Howlin & Udwin, 2006, Elison S., 2010), los problemas de estas personas en España son similares. En relación a las mediciones antropométricas, el 32% tiene un Índice de Masa Corporal (IMC) compatible con sobrepeso y el 18 % con obesidad. El 65-75% de los casos han tenido algún tipo de alteración cardiovascular, como estenosis aórtica supravalvular o estenosis periférica pulmonar, el 40% presentan hipertensión franca, y un 25% tienen cifras de tensión arterial en el límite superior de la normalidad. Además, muchos tienen síntomas compatibles con disfunción vesical (ir al lavabo con mucha frecuencia por el día o por la noche, urgencia urinaria) que puede requerir tratamiento farmacológico. Otro síntoma frecuente (50%) es el estreñimiento, en algunos casos acompañado de complicaciones (hemorroides, fisuras anales). Dos terceras partes (66%) presentan problemas osteoarticulares con afectación preferentemente de la columna vertebral. Desde el punto de vista neuroconductual, la mayoría de casos refieren ansiedad, obsesiones y fobias simples como los trastornos más frecuentes, en algunos de ellos afectando de manera importante su calidad de vida.

Por tanto, dada la elevada incidencia de diversas manifestaciones clínicas en los adultos con síndrome de Williams, muchas de ellas serias pero tratables, es importante enfatizar en la necesidad de un seguimiento clínico organizado para estas personas. En el caso del síndrome de Williams, existe una guía clínica publicada en el 2010 en la revista de la Sociedad Española de Pediatría y también en la revista de la Asociación, que indica de manera clara el protocolo de seguimiento recomendado para cada paciente por los diferentes especialistas según los diversos rangos etarios (del Campo Casanelles y Pérez-Jurado, 2010).

Además, existen unas recomendaciones médicas y de salud general que se deben transmitir a las familias para anticipar y minimizar los posibles problemas médicos y conductuales en los adultos con síndrome de Williams:

- Es muy importante el control de la ganancia de peso mediante cambios en los hábitos alimenticios y actividad física. Es fundamental para prevenir posibles complicaciones asociadas al síndrome de Williams, tanto cardiológicas, endocrinológicas (posible intolerancia a la glucosa y riesgo de diabetes) y del aparato locomotor (rodilla y columna) y digestivo (estreñimiento crónico). Se recomienda una **dieta equilibrada** y **baja en calorías** con alto contenido de frutas y verduras, así como evitar bebidas con cafeína y alto contenido de azúcar. De la misma manera, conviene

mantener una **actividad física mantenida**, con la práctica de algún deporte (natación, otros) y caminando al menos 30 minutos tres veces a la semana

- Entre las diversas evaluaciones preferiblemente multidisciplinares, se recomienda mantener una **evaluación cardiológica periódica**. La frecuencia de las evaluaciones se puede espaciar dependiendo de la presencia o no de problemas detectables, pero no se debe dar nunca el alta definitiva. Igualmente, se recomienda realizar **controles periódicos de la tensión arterial** y registrarlos.
- Se recomienda además realizar una **evaluación audiológica** de control para descartar posible pérdida auditiva y una **prueba de tolerancia oral a la glucosa** a partir de los 30 años .
- En relación a la urgencia urinaria ocasional, se recomienda realizar cambios con respecto a los **hábitos de ingesta de líquidos** (por ejemplo: evitar la ingesta en las últimas 2 horas antes de acostarse) y en los **hábitos miccionales** (por ejemplo: ir al lavabo de manera frecuente y con horario establecido, antes de viajes o actividades concretas, etc).
- Si existe trastorno de ansiedad que no remite y tiende a generalizarse, se debe intervenir de manera individualizada, dado que no está establecido cuál es el mejor protocolo de manejo. Se recomienda **apoyo psicológico** y realizar **técnicas de relajación** y **auto-control** que siempre son beneficiosas, aunque puede precisarse medicación en algunos casos.

El cuidado de los adultos también deberá estar enfocado a las necesidades que presentan con relación a su auto-cuidado, autonomía, interacciones sociales y situación educativa o laboral. Se estima que aproximadamente el 75% de los adultos requieren nula o mínima ayuda con las tareas de auto-cuidado y aproximadamente el 20% son capaces de llevar a cabo las tareas domésticas (Elison S., 2010). En nuestra serie, la mayoría requerían nula o mínima ayuda con las tareas de auto-cuidado, el 73% tienen cierto grado de autonomía doméstica y el 50% tienen cierta autonomía de desplazamiento, capaces de movilizarse en cortas distancias (Howlin & Udwin, 2006, Elison S., 2010). Es muy importante trabajar desde edades tempranas los diferentes aspectos de la vida cotidiana con la finalidad de que se logre la mayor autonomía posible.

A pesar de que las personas con síndrome de Williams tienen una personalidad muy sociable, es un hecho que tienen problemas para entablar y mantener amistades cuando alcanzan la adolescencia y

edad adulta, y El mismo estudio mencionado anteriormente encontró que aproximadamente el 40% de los pacientes tenían buenas amistades (Elison S., 2010). Con la finalidad de conocer a gente nueva se puede promover que asista a actividades de ocio o a las actividades organizadas por la Asociación de Síndrome de Williams. Para mantener amistades se puede promover su participación de manera supervisada en redes sociales (Facebook, Tuenti) y/o correos electrónicos

No podemos dejar de lado la importancia que el paciente alcance la máxima integración laboral posible. Se debe continuar con un programa formativo individualizado, que incluya terapia ocupacional, cursos de formación, voluntariado o trabajo remunerado.

Por último, otro tema que se debe mencionar durante la consulta es la sexualidad. Algunos autores sugieren comenzar a discutir el tema desde temprana edad para que sea más cómodo y facilitar el proceso de comunicación en la familia (Murphy & Elias, 2006). Es muy importante que reciban una adecuada educación sexual, ya que las personas con síndrome de Williams son potencialmente fértiles y con normal capacidad de funcionamiento en la vida sexual. En este sentido, es importante también tratar el tema de las dificultades para asumir una parentalidad responsable. Desde el punto de vista de asesoramiento genético, se debe informar acerca del riesgo de transmisión que existe. Los personas con síndrome de Williams tienen un 50% de probabilidad de transmitir su condición a sus descendientes.

En conclusión, la evaluación global del paciente con síndrome de Williams adulto debería ser idealmente en consultas multidisciplinares y por profesionales con experiencia, siendo también fundamental la figura del médico primario responsable del seguimiento habitual en interacción ocasional con los especialistas. Esto es difícil de conseguir en muchos lugares, y en la mayoría de casos el seguimiento por diversos profesionales se hace de manera independiente. No obstante, independientemente de las circunstancias hay que intentar una adecuada translación y mantener un seguimiento de los adultos de por vida, basado en la historia clínica previa y los protocolos establecidos.

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Pauta de Seguimiento en Adultos con Síndrome de Williams

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El síndrome de Williams Beuren (SWB) es un trastorno del neurodesarrollo causado por una deleción heterocigota en el cromosoma 7, concretamente en la región 7q11.23. Este síndrome cursa con una afectación multisistémica que incluye rasgos faciales típicos, alteraciones cardiovasculares (como estenosis de medianos y grandes vasos, hipertensión arterial), alteraciones endocrinas (intolerancia a la glucosa, hipercalcemia transitoria, discapacidad intelectual) y un fenotipo conductual asimétrico caracterizado por preservación relativa de las habilidades verbales y dificultades visuoespaciales.

Debido a la afectación de múltiples órganos y sistemas, los pacientes con síndrome de Williams deberían realizar un seguimiento periódico en consultas compuestas por diversos especialistas como genetistas clínicos, cardiólogos, nefrólogos, asesores genéticos, psicólogos, entre otros. El manejo de los pacientes pediátricos suele ser llevaba a cabo por el pediatra, quién se encarga de coordinar el manejo y seguimiento por los diferentes profesionales. En el 2001 el Comité de Genética de la Asociación Americana de Pediatría (Committee on Genetics, 2001) publicó unas pautas de seguimiento para pacientes pediátricos.

Gracias al aumento de conocimiento de la historia natural del síndrome de Williams, se conoce que durante la etapa adulta continúan algunas de las manifestaciones clínicas y pueden aparecer nuevos síntomas y complicaciones. A partir de varios estudios realizados en adultos con síndrome de Williams enfocados en diferentes sistemas (Bedeschi et al., 2011; Cherniske et al., 2004; B R Pober et al., 2010), se han escrito una serie de recomendaciones para su seguimiento médico y psicosocial (Howlin & Udwin, 2006; Barbara R Pober & Morris, 2007; Stinton, Tomlinson, & Estes, n.d.).

En el 2010 se publicó un protocolo de seguimiento en la revista de la Sociedad Española de Pediatría que indica de manera clara el seguimiento que debe realizar la persona con SWB por los diversos especialistas según rangos etarios (del Campo Casanelles & Pérez-Jurado, 2010). A continuación detallamos el esquema de

seguimiento para pacientes adultos, así como una tabla anexa que podrá ayudar a las familias a llevar un registro de las diferentes evaluaciones médicas recomendadas.

Mayores de 18 años:

El seguimiento debe continuarse con controles similares en la vida adulta, más dirigidos a los nuevos síntomas y complicaciones si se desarrollan.

Sería recomendable que las personas con síndrome de Williams mayores 18 años sean visitados cada dos años por un equipo **multidisciplar**, que incluye la evaluación por diferentes profesionales. Las evaluaciones que se precisan serán al menos las siguientes:

- **Historia clínica detalla**, incluyendo aspectos médicos relevantes que han ocurrido desde la última visita médica.
- **Evaluación de medidas antropométricas** para el control de la ganancia de peso (peso, talla, índice de masa corporal).
- **Examen físico completo,** incluye toma de signos vitales (frecuencia cardiaca, tensión arterial).
- Evaluación **oftalmológica** para descartar errores refractarios y cataratas.
- Evaluación **cardiológica**, incluye el control de la tensión arterial.
- Evaluación **ortopédica**, para evaluar alteraciones de la curvatura de la columna.
- Evaluación **endocrinológica,** para descartar problemas tiroideos o del metabolismo de glucosa.
- Controlar el **hábito intestinal**, para descartar problemas de estreñimiento, hemorroides y/o prolapso rectal.
- Evaluación del sistema **urogenital** para descartar problemas de incontinencia urinaria.
- Apoyo **psicológico** a la persona y a su familia.
- Valoración psicológica o **psiquiátrica** si hay problemas serios (con afectación de las actividades diarias).

Las personas que presenten problemas médicos en más de un aparato o sistema podrían ser visitados con mayor frecuencia (cada año) por el mismo equipo multidisciplinar.

Sería recomendado que aquellas personas que presenten un problema médico realicen un seguimiento con mayor periodicidad determinado por su especialista.

Evaluaciones complementarias

- Evaluación **audiológica** basal a partir de los 30 años y evaluaciones periódicas cada 5 – 10 años para descartar pérdida auditiva y prevenir acumulación de cera.

- Evaluaciones **odontoestomatológicas** según requiera.
- Ecografía de **riñones** y vejiga cada 5 – 10 años o si presenta síntomas.
- Se puede repetir el estudio de la **función renal** y del **metabolismo del calcio** cada 4 años.
- Se puede realizar pruebas de **función tiroidea** y niveles de TSH cada 3 años.
- Se puede realizar una prueba de tolerancia oral a la **glucosa** a partir de los 30 años y repetir cada 5 años o si presenta síntomas.

Recomendaciones Generales

- Control de la ganancia de peso mediante hábitos de vida saludables. Mantener una dieta equilibrada, con alto contenido de frutas y verduras. Evitar bebidas con alto contenido de azúcar.
- Realizar actividad física, caminar al menos 30 minutos al día, tres veces a la semana.
- Se debe mantener un programa de **estimulación** cognitiva y seguimiento.
- Continuar con el trabajo de los diferentes aspectos de la autonomía (doméstica, económica y de desplazamiento).
- Ayuda a la integración social y laboral, con el objetivo de conseguir una actividad laboral estable (más o menos supervisada), al menos a tiempo parcial.
- Importante mantener actividades de ocio.
- Trabajar para conseguir una vida independiente (valoración de vivir en pisos tutelados).

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